**Distribution Agreement**

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Elizabeth O'Brien Stenger Date

Expansion, Phenotype, and Function of Bone Marrow-Derived Mesenchymal Stromal Cells from Individuals with Sickle Cell Disease

By

Elizabeth O'Brien Stenger, MD
Master of Science in Clinical Research

_____

Jacques Galipeau, MD FRCP(C)
Advisor

_____

Mitch Klein, PhD
Committee Member

_____

Muna Qayed, MD MSc
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

# Expansion, Phenotype, and Function of Bone Marrow-Derived Mesenchymal Stromal Cells from Individuals with Sickle Cell Disease

By

Elizabeth O'Brien Stenger
B.S., University of Notre Dame, 2002
M.D., University of Pittsburgh School of Medicine, 2006

Advisor:
Jacques Galipeau, MD FRCP(C)

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Research

2016

## Abstract

Expansion, Phenotype, and Function of Bone Marrow-Derived Mesenchymal Stromal Cells from Individuals with Sickle Cell Disease

By
Elizabeth O'Brien Stenger

Hematopoietic cell transplantation (HCT) is the only curative therapy for sickle cell disease (SCD), but for the majority of patients who lack a matched donor, engraftment remains a significant barrier. Based upon dual function in promoting hematopoiesis and immunomodulation, mesenchymal stromal cells (MSCs) are an attractive cell-based therapy to modulate immunity and engraftment post-HCT. Studies have revealed limitations in approaches using cryopreserved, random donor MSCs, suggesting that fresh, autologous MSCs could circumvent these limitations. MSCs were expanded *ex vivo* from bone marrow (BM) samples from pediatric SCD patients and healthy adult volunteers. Doubling time of SCD MSCs was comparable to non-SCD MSCs. Phenotype of SCD MSCs, including flow cytometry for MSC surface markers and expression of indoleamine 2,3-dioxygenase (IDO) by RT-PCR, did not differ significantly to non-SCD MSCs. Non-SCD and SCD MSCs suppressed third-party and autologous (SCD) T cell proliferation in a dose-dependent manner. Expression of 47 hematopoiesis genes by Fluidigm array demonstrated minor differences by donor source and the potential to augment MSC expression by cytokine stimulation. These data demonstrate the feasibility of expanding autologous BM-derived MSCs from SCD patients. Importantly, phenotype and function of SCD MSCs are comparable to non-MSCs, supporting the use of autologous MSCs to enhance engraftment in SCD patients following haploidentical HCT.

# Expansion, Phenotype, and Function of Bone Marrow-Derived Mesenchymal Stromal Cells from Individuals with Sickle Cell Disease

By

Elizabeth O'Brien Stenger
B.S., University of Notre Dame, 2002
M.D., University of Pittsburgh School of Medicine, 2006

Advisor:
Jacques Galipeau, MD FRCP(C)

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Research

2016

**TABLE OF CONTENTS**

## LIST OF TABLES AND FIGURES

# INTRODUCTION

Approximately 100,000 Americans and millions of individuals worldwide have sickle cell disease (SCD) (1, 2), and despite advances in medical treatment such as hydroxyurea and chronic transfusion, individuals with SCD have significant morbidity and early mortality (3, 4). Hematopoietic cell transplantation (HCT) is currently the only cure, with excellent survival following human leukocyte antigen (HLA)-matched sibling donor transplant (5); however, fewer than 18% have an appropriate matched sibling donor (6). There is a paucity of HLA-matched unrelated donors for blacks in registries (7), leaving haploidentical donors (typically the subject's mother) an attractive option. The use of alternative donors has expanded the use of HCT, but at the expense of graft failure mediated predominantly by residual recipient T-lymphocytes (8-10). Engraftment failure is one of the most important barriers to successful cure of SCD with HCT and leads to a significant unmet clinical need.

The critical role of recipient T lymphocytes in graft rejection highlights the need for novel T lymphocyte directed therapies. Based upon their immunoregulatory properties (11, 12) and role in promoting hematopoiesis within the normal bone marrow niche, mesenchymal stromal cells (MSCs) are an attractive cell-based therapy to modulate the immune system following HCT. MSCs can enhance murine HSC engraftment, and several clinical trials demonstrated promising results when MSCs were given peri-transplant (13-17). A small pilot study of MSCs in pediatric patients with hemoglobinopathies undergoing unrelated donor HCT had no MSC safety concerns but was halted prematurely due to high transplant-related mortality (18); this study does highlight factors that may account for differences in MSC efficacy between pre-clinical

and clinical studies, including the use of third-party, cryopreserved MSCs. Although MSCs have been described as immunoprivileged, pre-clinical studies suggest that MSC donor source (19, 20) and culture conditions (21) alter their immunoregulatory potential. Differences in MSC clinical efficacy may also relate to the mechanisms of graft failure in different donor settings- HSC homing or cell number is likely more important in cord blood transplant, whereas graft failure following haploidentical transplant is predominantly immune-mediated (13). This suggests that MSCs could be particularly efficacious in promoting engraftment of haploidentical HSCs.

The objectives of this study were to verify the ability to *ex vivo* expand MSCs from the bone marrow (BM) of pediatric patients with SCD and to confirm MSC phenotype and function. We hypothesized that MSCs from patients with SCD could be expanded to clinically relevant dosages within 2-3 weeks, the time period between autologous back-up BM harvest (as a safety in the case of graft failure without autologous reconstitution) and planned infusion of MSCs post-haploidentical stem cell transplantation. We further hypothesized that MSCs from SCD patients would have phenotypical and functional properties comparable to MSCs from healthy non-SCD individuals. Novel experiments were performed to assess the impact of cytokine priming on MSC support of hematopoiesis and to confirm enhancement of immune modulation (following cytokine priming) in MSCs obtained from patients with SCD. These studies will be critical to garner Investigation New Drug (IND) support for a proposed trial of autologous MSCs to improve engraftment of haploidentical HSCs in SCD patients. These investigations could also provide evidence that autologous MSCs are superior to human

leukocyte antigen (HLA)-unmatched MCSs cryopreserved via standard techniques and evidence of pharmacological superiority of cytokine licensed MSCs.

## BACKGROUND

SCD, which is caused by a mutation in the red blood cell β-globin gene, is associated with substantial morbidity and risk of premature mortality from recurrent vaso-occlusion and ischemia. There are an estimated 100,000 Americans (1) and millions of individuals in Asia and Africa (2) living with SCD, leading to a significant burden of disease. Medical treatment with hydroxyurea can decrease morbidity from vaso-occlusive pain crises and acute chest syndrome, but it does not decrease the incidence of severe pain crises or recurrent stroke (4). Chronic transfusion therapy can decrease a number of complications by increasing normal hemoglobin levels relative to abnormal hemoglobin S. Approximately 10% of SCD patients have an overt stroke prior to age 20, and without chronic transfusion therapy, around 65% will have an additional stroke (3). However, despite chronic transfusion, one-fifth of pediatric patients have a second overt stroke, and there is a high incidence of progressive as well as new silent infarcts. Transfusion therapy also leads to complications from iron overload and alloimmunization. Despite these therapies, there is a steady decline in survival in patients with SCD observed by early adulthood (22). Patients with symptomatic SCD who participated in a Multicenter Study of Hydroxyurea closely resemble patients referred for HCT, and despite hydroxyurea therapy, they had an annual mortality rate of 4.4 per 100 person-years (23).

Pediatric patients with severe SCD have >90% overall survival and approximately 85% event-free survival following matched sibling donor transplant with standard myeloablative (full intensity) conditioning (5). Recent clinical trials have demonstrated that excellent outcomes can be extended to adult patients up to 40 years of age (24). A lack of suitably matched donors has, however, limited expansion of this curative

treatment to the majority of SCD patients. While one-quarter of patients should have a HLA-matched sibling, studies of children with SCD found that only 14% had an HLA-identical sibling (6). Other donor options include HLA-matched unrelated living donors or umbilical cord blood, but low numbers of black donors in the National Marrow Donor Program registry and a high degree of genetic polymorphisms limit unrelated donor options (7, 25, 26). As a result, only 19% of African Americans have an 8/8 HLA matched unrelated donor (25). Consequently, 66% of patients with SCD considered for HCT will be unable to undergo transplantation because of the lack of an available HLA-matched donor.

Engraftment of HSCs is a considerable barrier to successful cure of SCD with transplant, particularly in the HLA-mismatched setting. Early studies of matched sibling donor transplant with reduced-intensity conditioning demonstrated unacceptably high rates of graft loss (27). While studies of matched unrelated donor transplant following reduced-intensity conditioning are ongoing, particularly in young adults, cord blood engraftment continues to be challenging in these patients (28). Graft failure is predominantly immune-mediated, wherein recipient T cells play a dominant role in rejecting donor hematopoietic cells (10, 29). The risk of graft failure therefore increases with degree of HLA mismatch and decreased intensity of pre-transplant conditioning (10). SCD patients also have higher rates of graft failure due to a competent immune system, baseline inflammatory state, highly proliferative bone marrow, and alloimmunization from chronic transfusion (5, 28). For these reasons, graft rejection has been a significant obstacle to successful haploidentical transplant, particularly in SCD patients. The use of post-transplant cyclophosphamide has decreased graft-versus-host

disease (GVHD) after haploidentical transplant, based upon proliferating alloreactive T cells being more sensitive to cytotoxicity, but graft rejection remains a significant barrier (8, 9). While GVHD rates have been quite low using this regimen in patients (primarily adult) with SCD (9), graft failure remains extraordinarily high at approximately 40%, highlighting the need for novel interventions that target recipient T cells.

MSCs are pluripotent progenitors that are present in small numbers within normal bone marrow and that function in promoting hematopoiesis. They co-localize with HSCs in the bone marrow niche, producing factors that promote and recruit HSCs and regulate their function (11). They also have unique immunoregulatory properties, making them attractive for use as cell-based therapy to modulate the immune system, particularly following HCT. MSCs regulate both innate and adaptive immune responses through effects on various immune cells, particularly T cells and antigen-presenting cells (30-32). One of the major mechanisms by which MSCs down-regulate immune responses is via inhibition of cytotoxic T cell proliferation and function, which occurs by interferon (IFN)-γ-dependent upregulation of indoleamine 2,3-dioxygenase (IDO) (12).

A surge in pre-clinical and clinical studies of MSCs led the International Society for Cellular Therapy (ISCT) to define minimal phenotypic and functional criteria for MSCs, including plastic-adherence, pluripotent differentiation, expression of CD105, CD73, and CD90, and lack of hematopoietic antigen expression and HLA-DR (33, 34). Numerous clinical trials have demonstrated the feasibility of *ex vivo* expanding MSCs and safety following infusion, and there are currently more than 300 trials registered at www.clinicaltrials.gov. Pre-clinical studies demonstrate that MSCs can enhance donor HSC engraftment, and several clinical trials had promising results when MSCs were

given peri-transplant (13-17).

A single study has reported on the use of primarily third-party (random donor) cryopreserved MSCs to improve engraftment in pediatric patients undergoing HCT for hemoglobinopathies, including 4 patients with SCD, using a reduced intensity conditioning regimen (18). Two SCD patients had graft rejection with autologous reconstitution, both having undergone HLA-mismatched unrelated cord blood transplant. The other two SCD patients underwent HLA-mismatched unrelated bone marrow transplant, with both patients dying of transplant related complications (acute GVHD and CMV infection). Although the authors report no MSC safety concerns, the study was halted prematurely due to high transplant-related mortality, predominantly from infections. While the small number of patients and diverse donor HSC and MSC sources make it difficult to summarize the data, it is important to note that this group of patients was at very high risk for graft failure due to source of donor HSCs (all were from mismatched unrelated donors with a predominance of umbilical cord blood) and alloimmunization, with 5 of 6 patients on chronic transfusions. This study does also highlight some factors that may account for differences in MSC efficacy between pre-clinical and clinical studies, including the use of third-party, cryopreserved MSCs, which have been the largest source in clinical trials.

Although MSCs have been described as immunoprivileged, pre-clinical studies suggest that MSC donor source and culture conditions alter their immunoregulatory potential. MSCs upregulate major histocompatibility complex (MHC) class I and express MHC class II under inflammatory conditions, and MHC-mismatched murine MSCs undergo specific immune-mediated rejection (19). MSC donor source has been shown to

significantly impact the outcome of transplant in a non-myeloablative experimental model, where infusion of donor MSCs stimulated immune-mediated graft rejection, host MSCs promoted engraftment, and third-party MSCs had no effect (hypothesized by the authors to be due to their rejection) (20). These pre-clinical studies suggest that, despite the majority of clinical trials to date ignoring MHC barriers through the use of allogeneic or random donor MSCs, the immunology of MSCs very much matters. MSC donor source may be the single most important factor accounting for differences seen in pre-clinical and clinical trials, including studies of MSCs to treat GVHD. Further, clinical studies demonstrate that the bone marrow MSC compartment remains recipient following allogeneic HCT (35-38), which strongly suggests that the biology of autologous MSCs (and resultant tolerance) is distinct from random donor or donor-derived MSCs.

The majority of clinical trials have also used cryopreserved, serially expanded MSCs. Freshly thawed MSCs have impaired *in vitro* T cell suppression (with diminished IFN-γ-dependent IDO up-regulation) secondary to a reversible heat shock response, which can be restored following a 24-48 hour culture recovery period (39). Serial expansion of MSCs results in clonal impoverishment, telomere shortening, and increased cell senescence, and MSC passage has been shown to correlate with response and survival from acute GVHD, suggesting a relationship with functional impairment (21, 40). The immunoregulatory potential of MSCs is also dependent on IFN-γ being present in the microenvironment, and MSCs pre-licensed in IFN-γ have enhanced inhibition of T cell proliferation and function (41). Such factors may have contributed to the negative phase III trial of MSCs in the treatment of steroid-refractory acute GVHD, where efficacy had been seen in pre-clinical and early clinical trials (21).

In the setting of MSCs to promote engraftment, the clinical efficacy of such an approach may relate to the mechanisms of graft failure in different donor settings. HSC homing or cell number is likely more important in umbilical cord blood transplant, whereas graft failure following haploidentical transplant is predominantly immune-mediated (13). This suggests that MSCs could be particularly efficacious in promoting engraftment of haploidentical HSCs. The proposed studies may help to resolve the inconsistencies observed between pre-clinical and clinical studies as well as support the rationale for using autologous MSCs that undergo a culture recovery period (following cryopreservation) prior to infusion.

# METHODS

## Research objectives

As MSCs from individuals with SCD had not previously been evaluated, the primary objectives of this research were to evaluate the growth, phenotype, and function of these MSCs according to standard lab-based assays (33) and to compare these measures to MSCs obtained from healthy individuals without SCD. While equivalency was not formally evaluated in this research, the research was based upon the hypothesis that MSCs from patients with SCD are equivalent to non-SCD MSCs according to standard assays, including those put forth by the ISCT (33). While intrinsically interesting, the penultimate goal of these experiments was to justify the use of autologous MSCs (versus allogeneic or third party, as the majority of clinical trials have utilized) to promote engraftment of haploidentical HSCs in patients with SCD undergoing HCT as curative therapy. Prior to the *in vivo* use of SCD MSCs, it was necessary to demonstrate that they could be expanded *ex vivo* from BM samples (including growth as measured by MSC doubling time) and that their phenotype (expression of cell surface antigens characteristic of MSCs and as measured by flow cytometry) and function (expression of IDO as measured by RT-PCR and ability to suppress T cell proliferation as measured by flow cytometry following MSC co-culture) was consistent with standard assays. Lastly, the objectives of the final experiments performed and described (e.g. Fluidigm array) were to demonstrate the ability to use this approach to evaluate MSC expression of genes involved in hematopoiesis and to generate hypotheses for follow-up experiments utilizing alternative cytokine stimulation strategies (beyond IFN-γ) to augment MSC expression of

hematopoiesis genes, which may generate a MSC product superior to unstimulated MSCs for enhancing HSC engraftment *in vivo*.

**Study design and characteristics of the study population**

Following IRB approval and informed consent, MSC were isolated from BM aspirated from the posterior iliac crest of healthy adult volunteers (n=9) and from pediatric patients with SCD (n=8). The patients with SCD were recruited through the Aflac Blood and Cancer Disorders Center Blood and Marrow Transplant Program prior to undergoing (prospective fresh samples) or following (retrospective cryopreserved samples) matched sibling donor transplant. BM samples were all obtained at the time of autologous back-up BM harvest performed prior to matched sibling donor transplant as standard of care. Fresh BM samples were obtained from six of the SCD patients (6-10 mL) and cryopreserved BM samples were obtained from the remaining two SCD patients (2 mL post-Ficoll). Cryopreserved samples were obtained from the Stem Cell Laboratory at Children's Healthcare of Atlanta, where they had been initially stored in cryovials as part of routine care. Fresh BM samples were obtained from all healthy volunteers (10-20 mL) who were recruited through Emory University.

**Measurements**

**MSC isolation and culture**

Bone marrow aspirates were diluted 1:2 with phosphate-buffered saline and layered unto a Ficoll density gradient. The cells were centrifuged 400 g for 20 minutes and thereafter the mononuclear cells were plated in complete human MSC medium (α-MEM, 10%

human platelet lysate [hPL] (42) or 10% fetal bovine serum [FBS], and 100 U/ml penicillin/streptomycin) at 100,000-300,000 cell/$cm^2$. Non-adherent hematopoietic cells were removed by changing the medium after approximately 3 days of culture, and then MSCs were allowed to expand for 7-12 days (Passage 0, P0). Thereafter, the cells were passaged approximately every 7 days by treatment with trypsin/ ethylenediaminetetraacetate (EDTA) and reseeded in fresh MSC medium at 1000 cells/$cm^2$. MSCs were counted at P0 and P1 using Invitrogen™ Countess™ automated cell counter (Invitrogen Grand Island, NY USA).

**Phenotyping of MSCs by flow cytometry**

MSCs underwent flow cytometric analysis for the expression of CD19, CD34, CD44, CD45, CD73, CD90, CD105, HLA-I, and HLA-DR (BD BioSciences, St Jose, CA). All samples were run on a Canto II flow cytometer (Beckman Coulter, Indianapolis, IN) with the appropriate isotype controls. Flow Jo software (Tree Star, Portland, OR) was used to calculate mean fluorescence intensity (MFI) and to generate histograms for the expression of each marker.

**Real time quantitative PCR**

MSCs were cultured for 6 days and activated for 48h with 20 ng/ml recombinant human IFN-γ. DNA-free total RNA was extracted and reverse transcribed as previously described (43). Real-time (RT)-qPCR assays were performed in duplicate on an ABI 7500 Fast Real-Time PCR system thermal cycler and SYBR Green Mastermix (Applied Biosystems) with human primer sequences for IDO and B-actin. Primers were designed

using the NCIB/Primer Blast designing tool (http://www-ncbi-nlm-nih-gov.proxy.library.emory.edu/tools/primer-blast/). Data was analyzed using the relative quantification method (44).

**T proliferation assay**

MSCs and T cells were co-cultured as previously described (41). In brief, MSCs were seeded at 5000 cm/cm$^2$ on a 96-well plate then remained either unstimulated or underwent IFN-γ stimulation (48 hrs, 20 ng/ml). Blood was obtained from healthy volunteers after informed consent on an IRB-approved protocol, after which peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient. PBMCs were cultured at 100,000 cells/well. T lymphocytes were stimulated using anti-CD3/anti-CD28 Dynabeads (Invitrogen, Carlsbad, CA). Ki67 proliferation assay was performed after 4 days according to manufacturer instruction (BD Biosciences, San Jose, CA).

**Fluidigm nanoscale PCR array**

Quantitative RT-PCR was performed using a Fluidigm 48x48 nanofluidic array (45) that targeted 47 genes important in hematopoiesis (plus IDO as an internal positive control). In brief, RNA was extracted from MSCs ±IFN-γ stimulation (20 ng/ml), then converted to cDNA using polyT priming. The 48 targeted genes were pre-amplified in a single 14 cycle PCR reaction after combining 100 ng cDNA with pooled primers and TaqMan Pre-Amp Mastermix as described in the manufacturer's protocol (Fluidigm BioMark™, San Francisco, CA). Two thousand three hundred and four parallel qRT-PCR reactions were performed for each primer pair on each sample on a 48x48 array. Amplification was

detected using the EvaGreen detection assay on a Biomark I machine and following standard Fluidigm protocols. PCR data was normalized and analyzed with SAS/JMP Genomics software (Cary, NC).

**Statistical plan**

Patient clinical and BM characteristics (age, pre-HCT treatment with hydroxyurea, weight on day of HCT, volume of BM, source of BM [fresh versus frozen], and MNC count) were described in tabular form. Descriptive statistics (mean±standard deviation) were calculated for each variable by source of MSCs (SCD, non-SCD), including doubling time, fluorescence intensity of cell surface proteins, fold induction of IDO gene expression, percentage of $Ki67^+$ T cells ($CD3^+$), and cycle threshold of hematopoiesis genes. Mean values for SCD MSCs were compared to non-SCD MSCs using two sample t test and Wilcoxon rank sum test. Calculations were carried out using GraphPad Prism software (GraphPad Software, La Jolla, CA). P-value $<0.05$ was considered statistically significant. P-values reported are from two sample t test unless otherwise indicated.

Statistical analysis of Fluidigm data was performed using SAS JMP Genomics (Cary, NC). We first removed 10 probes based on the requirement that signal was detected (threshold Ct <30, the total number of cycles) in at least 20 percent of the samples. Each sample profile consisting of 38 transcripts was median-centered to statistically remove overall effects of overall RNA quantity. Multivariate analysis of variance was performed for each probe simultaneously fitting both IFN-γ stimulation status and SCD status as fixed effects. We used a simple approximate Bonferroni p-value cut-off of $p<0.001$ to adjust for the approximately 50 comparisons.

# RESULTS

## Characteristics of SCD patients and their bone marrow samples

We obtained 11 BM samples from patients with SCD prior to undergoing matched sibling donor BMT at Children's Healthcare of Atlanta's Aflac Cancer and Blood Disorders Center (Table 1). Patients ranged in age from 2-19 years (median, 8.3±4.7) and weighed 12.5-50.8 kg (median, 27.1±11.5). Eight of the 11 patients received hydroxyurea pre-HCT as medical treatment for their SCD, which was discontinued in all patients approximately 2 weeks prior to admission for transplant. Nine patients were consented for fresh samples while 2 patients were consented for frozen samples. For fresh samples, the starting mononuclear cell (MNC) count was $49.7x10^6 \pm 26.5x10^6$ in BM samples ranging from 6-10 ml.

## Growth expansion and cell surface phenotype of SCD MSCs compared to non-SCD MSCs

SCD MSCs were expanded to P1 for a total of 14.4±2.5 days. Growth of SCD MSCs (n=10) was compared to the expansion of MSCs from individuals without SCD (non-SCD; n=5). Time in hours for MSCs to double (P0 to P1; doubling time) was calculated and was comparable between SCD (34.3±10.6 hours) and non-SCD (44.1±10.4 hours) samples (p=0.11; Figure 1).

As per the ISCT guidelines (46), we confirmed MSC phenotype in non-SCD versus SCD MSCs (n=5/group, Figures 2 [histogram plots] & 3 [mean fluorescence intensity, MFI]). Low passage (P1-P3) cryopreserved MSCs were cultured for 5-7 days then analyzed using flow cytometry for the cell surface expression of markers used to

identify MSCs. MSCs from SCD patients displayed a typical MSC phenotype with >95% of cells positive for CD44, CD73, CD90, CD105, and HLA-I and <5% of cells positive for CD34, CD45, CD19, and HLA-DR (hematopoietic antigens). MFI for each marker was compared between SCD and non-SCD samples, with higher MFI for HLA-I ($p=0.03$), CD73 ($p=0.04$), and CD90 ($p=0.01$) in non-SCD samples, otherwise no difference was seen between groups (CD105: $p=0.55$, CD45: $p=0.26$, CD34: $p=0.33$, CD19: $p=0.53$, CD116: $p=0.38$, HLA-DR: $p=0.05$).

**Response of SCD MSCs to interferon-gamma stimulation**

As MSCs licensed with IFN-γ have been demonstrated to have superior immunomodulatory function which is dependent upon the upregulation of indoleamine 2,3-dioxygenase (IDO) (41), we next compared IDO gene expression in unstimulated and IFN-γ stimulated MSCs (n=4-5/group). SCD and non-SCD MSCs were cultured overnight and then either left untreated or stimulated with IFN-γ for 4 hours. The level of IDO expression was analyzed by qRT-PCR and reported as fold induction (Figure 4). Unstimulated MSCs had negligible expression of IDO (non-SCD: 1.01±0.08, SCD: 1.01±0.07), whereas IFN-γ stimulation resulted in significant upregulation of IDO by both non-SCD (47887±8420) and SCD (22019±7879) MSCs (comparing stimulated and unstimulated, non-SCD: $p=0.03$, SCD: $p=0.01$).There was no difference in IDO expression between non-SCD and SCD MSCs (unstimulated: $p=0.92$; stimulated: $p=0.11$). This data was also confirmed in qRT-PCR experiments performed using Fluidigm array, where IDO was included as a positive control (described below, Figures 9 & 10). The difference in IDO fold induction between non-SCD and SCD MSCs was -

0.72, which was not statistically significant (p=0.12), whereas the difference in IDO fold induction was 34.83 comparing IFN-γ stimulated to unstimulated MSCs, which was significant ($p=1.49x10^{-35}$).

**Immunomodulatory function of SCD MSCs compared to non-SCD MSCs**

To evaluate the immunomodulatory function of SCD MSCs, SCD and non-SCD MSCs (n=5/group) were co-cultured for 4 days first with third-party PBMCs (single sample used for all experiments), with or without anti-CD3/CD28 co-stimulation, and T cell proliferation was assessed by flow cytometric analysis of Ki67 expression (%$CD3^+Ki67^+$ cells). Figure 5 demonstrates representative flow cytometry gating strategy for a single SCD and non-SCD sample. When PBMCs were unstimulated, $CD3^+$ T cells did not proliferate appreciably, including when co-cultured with MSCs (data not shown). Conversely, stimulation of PBMCs resulted in extensive proliferation of $CD3^+$ T cells, with 63.2% having high Ki67 expression (Figure 6, "No MSC" data point). When PBMCs were co-cultured with MSCs at varying concentrations (MSC:PBMC ratios of 1:20, 1:10, and 1:5), both SCD and non-SCD MSCs suppressed T cell proliferation in a dose-dependent manner (for SCD MSCs: p=0.02 comparing 1:20 to 1:10, p=0.01 comparing 1:10 to 1:5). SCD MSCs more potently suppressed T cell proliferation at all concentrations compared to non-SCD MSCs (1:20, p=0.03; 1:10, p=0.01; 1:5, p=0.03).

Given our mechanistic hypothesis that autologous MSCs could promote HSC engraftment through the inhibition of residual recipient T cells, it was crucial to demonstrate that SCD MSCs could inhibit autologous (e.g. from the same SCD patient) T cells *in vitro*. Consequently, SCD MSCs were next co-cultured for 4 days with PBMCs

from the same SCD donor (n=3 MSC and PBMC donor pairs), with or without anti-CD3/CD28 co-stimulation, and T cell proliferation was assessed again by flow cytometric analysis of Ki67 expression. Results were compared to experiments performed with non-SCD MSCs from a single donor (BMH21). As shown in Figure 7, all MSC samples suppressed the proliferation of SCD PBMCs in a dose-dependent manner. Statistical analysis was performed on combined data, wherein no difference was seen between SCD MSCs and the non-SCD MSC sample (1:20, p=0.17; 1:10, p=0.15; 1:5, p=0.34; 1:2.5, p=0.28).

**Expression of targeted hematopoiesis genes in MSCs**

Finally, we designed a 48-probe qPCR platform informed by the literature on normal hematopoiesis. Expression of each these 48 genes was assessed using Fluidigm array in unstimulated and IFN-γ stimulated MSCs from individuals with and without SCD (n=4-5/group). In Figure 8, cycle threshold (CT) for each gene in each MSC sample is depicted as a heat map, where red=high expression and blue=low expression. Cluster analysis was performed and demonstrated no clustering by MSC donor source (clustering predominantly by IFN-γ stimulation status; Figure 8). Only four hematopoiesis genes were found to be significantly differentially expressed in SCD versus non-SCD MSCs ($p<0.001$), although the magnitude of the difference was small (range -2.8 to +1.0; Volcano plot, Figure 9; raw data, Table 2). On the contrary, 23 hematopoiesis genes were found to be significantly differentially expressed in unstimulated versus INF-γ stimulated MSCs ($p<0.001$), with a wider magnitude of difference (range -4.7 to +18.7; Volcano plot, Figure 10; raw data, Table 3).

## DISCUSSION/CONCLUSIONS

In these *in vitro* studies, we verified that it is feasible to expand BM-derived MSCs from patients with SCD prior to undergoing HCT. Despite the majority of patients receiving treatment for SCD with hydroxyurea (stopped approximately 2 weeks prior to obtaining marrow sample), an immunosuppressive medication that is also marrow suppressive, the *ex vivo* expansion of SCD MSCs (e.g. doubling time) was no different than that of non-SCD MSCs. Further, comparison of SCD and non-SCD MSCs revealed no clinically significant differences in their phenotype (by flow cytometry for surface markers and upregulation of IDO following IFN-γ priming) or function (by suppression of T cell proliferation following co-culture). Important to our mechanistic hypothesis for the subsequent planned clinical trial, SCD appeared to equally suppress the proliferation of autologous T cells (e.g. derived from the same SCD donor as the MSCs) when compared to non-SCD MSCs. Finally, in novel hypothesis-generating experiments examining MSC expression of hematopoiesis genes, we demonstrated no clinically significant differences between SCD and non-SCD MSCs with differential expression of a small number of genes when comparing unstimulated and IFN-γ stimulated MSCs. These *in vitro* studies demonstrate the feasibility of *ex vivo* expanding functional BM-derived MSCs from SCD patients prior to HCT and support a planned phase I (safety and early efficacy) clinical trial of autologous MSCs to enhance haploidentical stem cell engraftment in SCD patients. While equivalency was not formally evaluated (e.g. power calculations not performed to assess equivalency), these results do suggest that the growth, phenotype, and function of SCD MSCs is equivalent to non-SCD MSCs by the standard assays used to perform these measurements.

To our knowledge, these studies are the first to evaluate MSCs from individuals with SCD. Given that SCD is caused by an inherited hemoglobin defect that is thus present only in red blood cells (RBCs), our findings support our hypothesis that MSCs from SCD patients are indistinguishable from healthy control MSCs in terms of standard *in vitro* phenotypic and functional assays. These results are in contrast to studies examining MSCs from patients with myelodysplastic syndrome (MDS, where there is ineffective production of cells produced in the BM, including RBCs), in which MSCs have been reported to be defective (47). Despite our hypothesis, it was clinically necessary to confirm these results prior to infusion of autologous MSCs in the aforementioned clinical trial.

Further, our evaluation of MSC gene expression confirms that SCD MSCs equivalently express genes critical for hematopoiesis, which may be mechanistically important for the MSC augmentation of HSC engraftment seen in pre-clinical studies (20, 48-58) and suggested by early phase clinical trials (14-18, 59-63). Studies of cytokine-stimulated MSCs (initially performed with IFN-γ) suggest that MSC expression of hematopoiesis genes may be augmented by this approach, which may allow the development of a superior MSC product for this indication. For example, IFN-γ priming of MSCs led to a 4.8-fold increase in the expression of CXC3L1, a chemokine found to be important for MSC homing to sites of ischemic brain injury (64) and to pancreatic islets (65) as well as found to be diminished in MDS MSCs (47).

As MSCs have never been evaluated in SCD patients, it was critical to confirm normal phenotype and function prior to their *in vivo* use. As described above, our findings are consistent with the defect in SCD being in the RBC and not in other cell

populations within the BM (such as white blood cells or the immune system), although differences in inflammatory pathways (such as serum cytokine levels) have been associated with clinical severity of SCD (66). Given the equivalent suppression of autologous T cell proliferation by SCD MSCs, this provides further support for the use of an autologous MSC product in this setting, based upon our mechanistic hypothesis that MSCs may promote HSC engraftment (at least in part) via suppression of residual recipient T cells that contribute to graft rejection. These studies have been used to provide critical support for a Food and Drug Administration (FDA) IND application for the use of autologous MSCs to promote engraftment following haploidentical HCT in SCD patients (approved March 2016). Although this study will primarily be a safety study, demonstration of early efficacy may support the use of autologous MSCs in a subsequent larger phase II clinical trial as well as with additional alternative donor sources (e.g. cord blood), where engraftment also remains a significant issue.

In addition to evaluating the feasibility and safety of autologous MSC infusion within the planned phase I trial, next steps include evaluating the *in vivo* effects of MSCs on immune reconstitution, engraftment, and GVHD post-HCT (secondary endpoints for efficacy on the clinical trial). *In vitro* evaluations of the MSC product are also planned, which may aid in the development of surrogate markers/assays of MSC potency that could be utilized in future studies. Finally, evaluations of alternative MSC cytokine-priming strategies (such as with TGF-β) are planned, with the goal to augment MSC expression of hematopoiesis genes that may enhance their *in vivo* engraftment potentiating effects and may garner additional support for the use of a cytokine-primed MSC product.

The primary limitation of this study was the small number of patient samples included in these *in vitro* evaluations, although based upon previous evaluations of MSCs from patients with Crohn's disease (67) and GVHD (35), we anticipate these studies will be sufficient to obtain FDA IND for our clinical trial. In addition, samples were obtained from primarily pediatric SCD patients (due to access to patients); based upon the defect in SCD being present in RBCs, we do not anticipate this will be a significant issue. Finally, we were limited in this study by the available and currently accepted assays to evaluate MSCs. We hope, as discussed above, to determine more uniform approaches to evaluate the potency of MSCs for *in vivo* use within the confines of our planned phase I clinical trial.

In conclusion, MSCs from individuals with SCD are consistent with the phenotype established by the ISCT (46) and have functional properties comparable to MSCs obtained from individuals without SCD. Importantly, we have demonstrated the feasibility of ex vivo expanding MSCs from the BM of SCD patients prior to HCT. These *in vitro* studies provide support for the use of autologous MSCs to promote engraftment of haploidentical HSCs in high risk SCD patients within the confines of a phase I clinical trial.

## REFERENCES


1. Hassell KL. Population estimates of sickle cell disease in the U.S. *American journal of preventive medicine* 2010;38(4 Suppl):S512-21.
2. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. *Blood* 2010;115(22):4331-6.
3. Hulbert ML, McKinstry RC, Lacey JL, et al. Silent cerebral infarcts occur despite regular blood transfusion therapy after first strokes in children with sickle cell disease. *Blood* 2011;117(3):772-9.
4. Alvarez O, Yovetich NA, Scott JP, et al. Pain and other non-neurological adverse events in children with sickle cell anemia and previous stroke who received hydroxyurea and phlebotomy or chronic transfusions and chelation: results from the SWiTCH clinical trial. *American journal of hematology* 2013;88(11):932-8.
5. Bernaudin F, Socie G, Kuentz M, et al. Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. *Blood* 2007;110(7):2749-56.
6. Walters MC, Patience M, Leisenring W, et al. Barriers to bone marrow transplantation for sickle cell anemia. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 1996;2(2):100-4.
7. Dew A, Collins D, Artz A, et al. Paucity of HLA-identical unrelated donors for African-Americans with hematologic malignancies: the need for new donor options. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2008;14(8):938-41.

8. Brodsky RA, Luznik L, Bolanos-Meade J, et al. Reduced intensity HLA-haploidentical BMT with post transplantation cyclophosphamide in nonmalignant hematologic diseases. *Bone marrow transplantation* 2008;42(8):523-7.

9. Bolanos-Meade J, Fuchs EJ, Luznik L, et al. HLA-haploidentical bone marrow transplantation with posttransplant cyclophosphamide expands the donor pool for patients with sickle cell disease. *Blood* 2012;120(22):4285-91.

10. Olsson R, Remberger M, Schaffer M, et al. Graft failure in the modern era of allogeneic hematopoietic SCT. *Bone marrow transplantation* 2013;48(4):537-43.

11. Le Blanc K, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. *Nature reviews Immunology* 2012;12(5):383-96.

12. Francois M, Romieu-Mourez R, Li M, et al. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Molecular therapy : the journal of the American Society of Gene Therapy* 2012;20(1):187-95.

13. Bernardo ME, Cometa AM, Locatelli F. Mesenchymal stromal cells: a novel and effective strategy for facilitating engraftment and accelerating hematopoietic recovery after transplantation? *Bone marrow transplantation* 2012;47(3):323-9.

14. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2000;18(2):307-16.

15. Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2005;11(5):389-98.

16. Ball LM, Bernardo ME, Roelofs H, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* 2007;110(7):2764-7.

17. Macmillan ML, Blazar BR, DeFor TE, et al. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial. *Bone marrow transplantation* 2009;43(6):447-54.

18. Kharbanda S, Smith AR, Hutchinson SK, et al. Unrelated donor allogeneic hematopoietic stem cell transplantation for patients with hemoglobinopathies using a reduced-intensity conditioning regimen and third-party mesenchymal stromal cells. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2014;20(4):581-6.

19. Eliopoulos N, Stagg J, Lejeune L, et al. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* 2005;106(13):4057-65.

20. Nauta AJ, Westerhuis G, Kruisselbrink AB, et al. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006;108(6):2114-20.

21. Galipeau J. The mesenchymal stromal cells dilemma--does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy* 2013;15(1):2-8.

22. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *The New England journal of medicine* 1994;330(23):1639-44.

23. Steinberg MH, McCarthy WF, Castro O, et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. *Am J Hematol* 2010;85(6):403-8.

24. Hsieh MM, Fitzhugh CD, Weitzel RP, et al. Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *Jama* 2014;312(1):48-56.

25. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *The New England journal of medicine* 2014;371(4):339-48.

26. Mentzer WC, Heller S, Pearle PR, et al. Availability of Related Donors for Bone-Marrow Transplantation in Sickle-Cell-Anemia. *Am J Pediat Hematol* 1994;16(1):27-9.

27. Iannone R, Casella JF, Fuchs EJ, et al. Results of minimally toxic nonmyeloablative transplantation in patients with sickle cell anemia and beta-thalassemia. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2003;9(8):519-28.

28. Kamani NR, Walters MC, Carter S, et al. Unrelated donor cord blood transplantation for children with severe sickle cell disease: results of one cohort from the phase II study from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2012;18(8):1265-72.

29. Mattsson J, Ringden O, Storb R. Graft Failure after Allogeneic Hematopoietic Cell Transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2008;14(Supplement 1):165-70.

30. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;101(9):3722-9.

31. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;105(4):1815-22.

32. Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;105(5):2214-9.

33. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8(4):315-7.

34. Sensebe L, Bourin P, Tarte K. Good manufacturing practices production of mesenchymal stem/stromal cells. *Human gene therapy* 2011;22(1):19-26.

35. Copland IB, Qayed M, Garcia MA, et al. Bone Marrow Mesenchymal Stromal Cells from Patients with Acute and Chronic Graft-versus-Host Disease Deploy Normal Phenotype, Differentiation Plasticity, and Immune-Suppressive Activity. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2015;21(5):934-40.

36. Bartsch K, Al-Ali H, Reinhardt A, et al. Mesenchymal stem cells remain host-derived independent of the source of the stem-cell graft and conditioning regimen used. *Transplantation* 2009;87(2):217-21.

37. Rieger K, Marinets O, Fietz T, et al. Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation. *Experimental hematology* 2005;33(5):605-11.

38. Wang J, Liu K, Lu DP. Mesenchymal stem cells in stem cell transplant recipients are damaged and remain of host origin. *International journal of hematology* 2005;82(2):152-8.

39. Francois M, Copland IB, Yuan S, et al. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy* 2012;14(2):147-52.

40. von Bahr L, Sundberg B, Lonnies L, et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2012;18(4):557-64.

41. Chinnadurai R, Copland IB, Patel SR, et al. IDO-independent suppression of T cell effector function by IFN-gamma-licensed human mesenchymal stromal cells. *J Immunol* 2014;192(4):1491-501.

42. Copland IB, Garcia MA, Waller EK, et al. The effect of platelet lysate fibrinogen on the functionality of MSCs in immunotherapy. *Biomaterials* 2013;34(32):7840-50.

43. Romieu-Mourez R, Francois M, Boivin MN, et al. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. *J Immunol* 2007;179(3):1549-58.

44. Romieu-Mourez R, Francois M, Boivin MN, et al. Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. *J Immunol* 2009;182(12):7963-73.

45. Nath AP, Arafat D, Gibson G. Using blood informative transcripts in geographical genomics: impact of lifestyle on gene expression in fijians. *Frontiers in genetics* 2012;3:243.

46. Krampera M, Galipeau J, Shi Y, et al. Immunological characterization of multipotent mesenchymal stromal cells--The International Society for Cellular Therapy (ISCT) working proposal. *Cytotherapy* 2013;15(9):1054-61.

47. Geyh S, Oz S, Cadeddu RP, et al. Insufficient stromal support in MDS results from molecular and functional deficits of mesenchymal stromal cells. *Leukemia* 2013;27(9):1841-51.

48. Noort WA, Kruisselbrink AB, in't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Experimental hematology* 2002;30(8):870-8.

49. in 't Anker PS, Noort WA, Kruisselbrink AB, et al. Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Experimental hematology* 2003;31(10):881-9.

50. Hiwase SD, Dyson PG, To LB, et al. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. *Stem Cells* 2009;27(9):2293-300.

51. Kim DW, Chung YJ, Kim TG, et al. Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. *Blood* 2004;103(5):1941-8.

52. Kuci S, Kuci Z, Kreyenberg H, et al. CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica* 2010;95(4):651-9.

53. Bensidhoum M, Chapel A, Francois S, et al. Homing of in vitro expanded Stro-1- or Stro-1+ human mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. *Blood* 2004;103(9):3313-9.

54. Angelopoulou M, Novelli E, Grove JE, et al. Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice. *Experimental hematology* 2003;31(5):413-20.

55. Almeida-Porada G, Porada CD, Tran N, et al. Cotransplantation of human stromal cell progenitors into preimmune fetal sheep results in early appearance of human donor cells in circulation and boosts cell levels in bone marrow at later time points after transplantation. *Blood* 2000;95(11):3620-7.

56. Mielcarek M, Storb R, Georges GE, et al. Mesenchymal stromal cells fail to prevent acute graft-versus-host disease and graft rejection after dog leukocyte antigen-haploidentical bone marrow transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2011;17(2):214-25.

57. Lee WS, Suzuki Y, Graves SS, et al. Canine bone marrow-derived mesenchymal stromal cells suppress alloreactive lymphocyte proliferation in vitro but fail to enhance engraftment in canine bone marrow transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2011;17(4):465-75.

58. Masuda S, Ageyama N, Shibata H, et al. Cotransplantation with MSCs improves engraftment of HSCs after autologous intra-bone marrow transplantation in nonhuman primates. *Experimental hematology* 2009;37(10):1250-7 e1.

59. Bernardo ME, Ball LM, Cometa AM, et al. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the

risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. *Bone marrow transplantation* 2011;46(2):200-7.

60. Gonzalo-Daganzo R, Regidor C, Martin-Donaire T, et al. Results of a pilot study on the use of third-party donor mesenchymal stromal cells in cord blood transplantation in adults. *Cytotherapy* 2009;11(3):278-88.

61. Le Blanc K, Samuelsson H, Gustafsson B, et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* 2007;21(8):1733-8.

62. Meuleman N, Tondreau T, Ahmad I, et al. Infusion of mesenchymal stromal cells can aid hematopoietic recovery following allogeneic hematopoietic stem cell myeloablative transplant: a pilot study. *Stem cells and development* 2009;18(9):1247-52.

63. Ning H, Yang F, Jiang M, et al. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* 2008;22(3):593-9.

64. Zhu J, Zhou Z, Liu Y, et al. Fractalkine and CX3CR1 are involved in the migration of intravenously grafted human bone marrow stromal cells toward ischemic brain lesion in rats. *Brain research* 2009;1287:173-83.

65. Sordi V, Malosio ML, Marchesi F, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005;106(2):419-27.

66. Sarray S, Saleh LR, Lisa Saldanha F, et al. Serum IL-6, IL-10, and TNFalpha levels in pediatric sickle cell disease patients during vasoocclusive crisis and steady state condition. *Cytokine* 2015;72(1):43-7.

67. Chinnadurai R, Copland IB, Ng S, et al. Mesenchymal Stromal Cells Derived From Crohn's Patients Deploy Indoleamine 2,3-dioxygenase-mediated Immune Suppression, Independent of Autophagy. *Molecular therapy : the journal of the American Society of Gene Therapy* 2015;23(7):1248-61.

**Table 1: Baseline Clinical and Bone Marrow Characteristics in Pediatric SCD Patients**

| Study ID | Age (yr) | Pre-HCT HU | Weight at HCT (kg) | Volume BM (ml) | Source | MNC count |
|---|---|---|---|---|---|---|
| SCD001 | 2.6 | Y | 12.5 | 10 | Fresh | - |
| SCD004 | 10.9 | Y | 38.5 | 6 | Fresh | $17 \times 10^6$ |
| SCD005 | 4.1 | Y | 14.7 | 7.5 | Fresh | $58.5 \times 10^6$ |
| SCD006 | 19.9 | Y | 50.8 | 10 | Fresh | $13 \times 10^6$ |
| SCD007 | 10.7 | Y | 33.2 | 10 | Fresh | $75 \times 10^6$ |
| SCD009 | 5.3 | N | 18.3 | 10 | Fresh | $89 \times 10^6$ |
| SCD010 | 8.39 | N | 34.8 | 10 | Fresh | $40 \times 10^6$ |
| SCD011 | 6.06 | N | 21.5 | 10 | Fresh | $60 \times 10^6$ |
| SCD012 | 7.48 | Y | 27.9 | 10 | Fresh | $45 \times 10^6$ |
| SCD002 | 5.6 | Y | 19.4 | 2 | Frozen, post-ficoll | $4 \times 10^6$ |
| SCD003 | 10.8 | Y | 26.4 | 2 | Frozen, post-ficoll | $3.5 \times 10^6$ |

MSCs indicates mesenchymal stromal cells; SCD, sickle cell disease; HCT, hematopoietic cell transplantation; HU, hydroxyurea; BM, bone marrow; MNC, mononuclear cell.

**Figure 1: Growth of MSCs derived from SCD (n=10) and non-SCD (n=5) individuals as Assessed by Time to Double from Passage 0 to Passage 1**

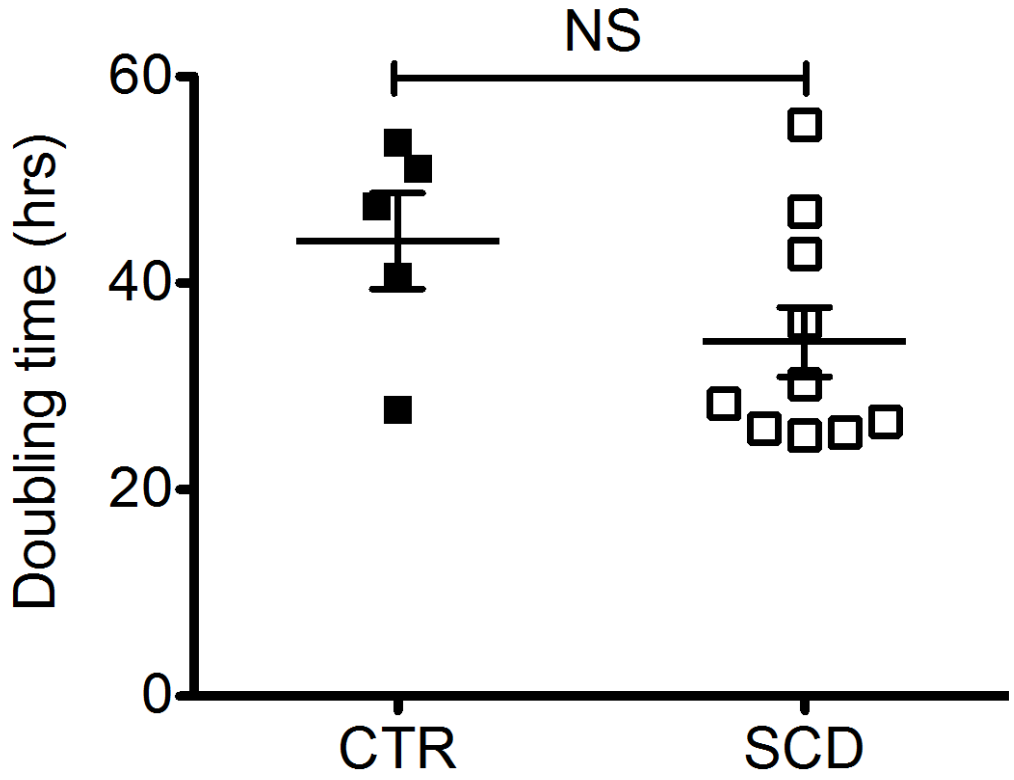
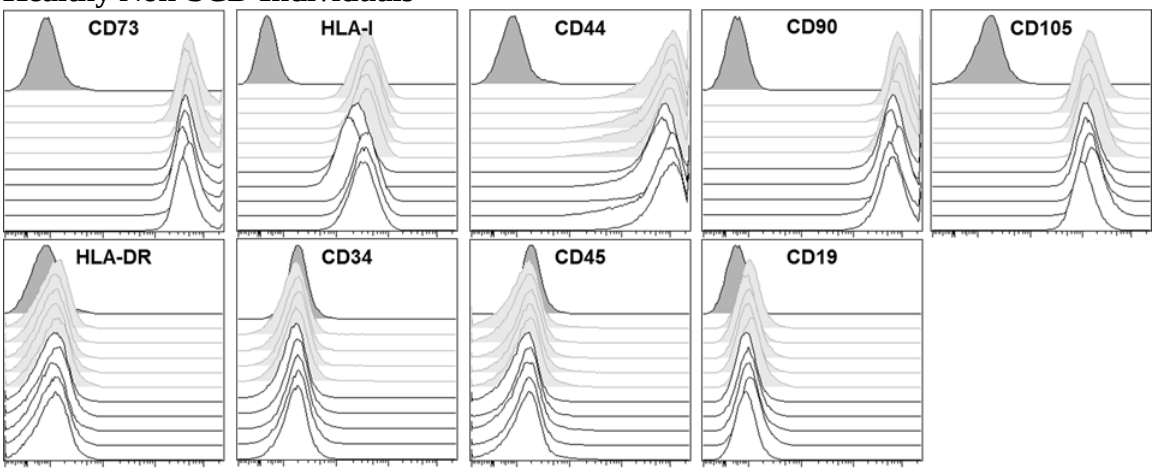


**Figure 2: Cell Surface Phenotype by Histogram of MSCs Derived from SCD and Healthy Non-SCD Individuals**




Light Grey: non-SCD, n=5
White: SCD, n=5
Dark Grey: unstained

**Figure 3: Cell Surface Phenotype by Mean Fluorescence Intensity of MSCs Derived from SCD and Healthy Non-SCD Individuals (n=5/group)**

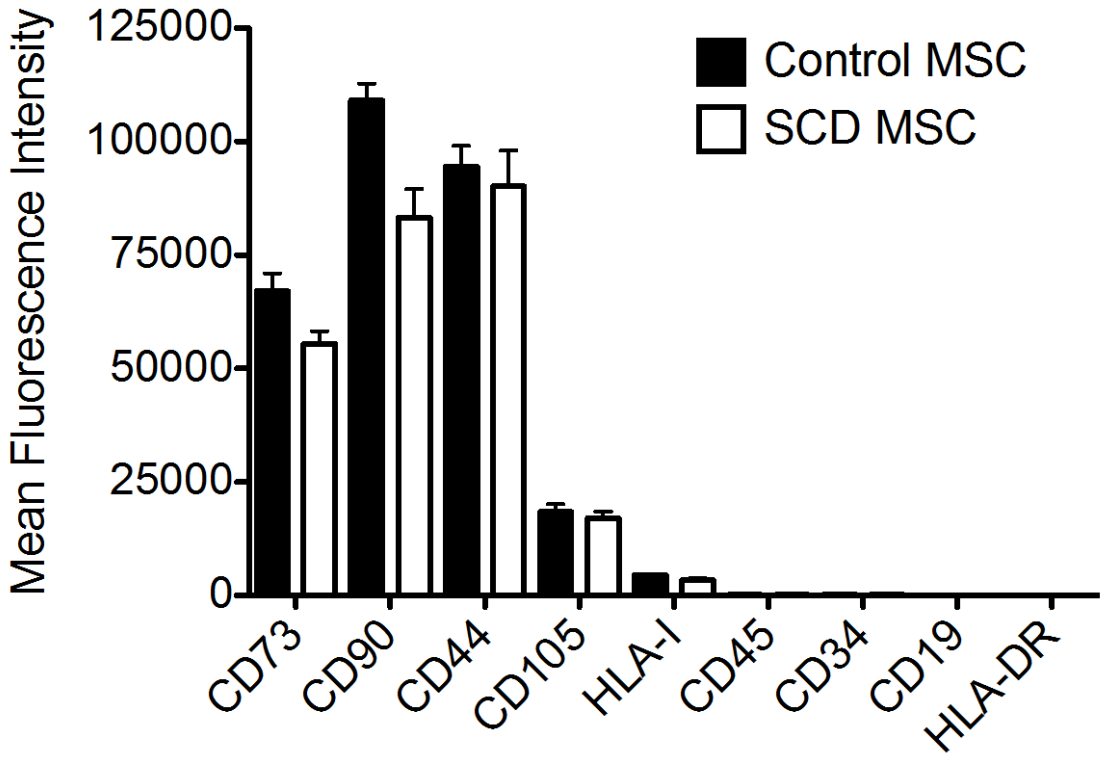


**Figure 4: Expression of IDO by Unstimulated and IFN-γ Stimulated SCD and non-SCD MSCs as Assessed by RT-PCR (n=5/group)**

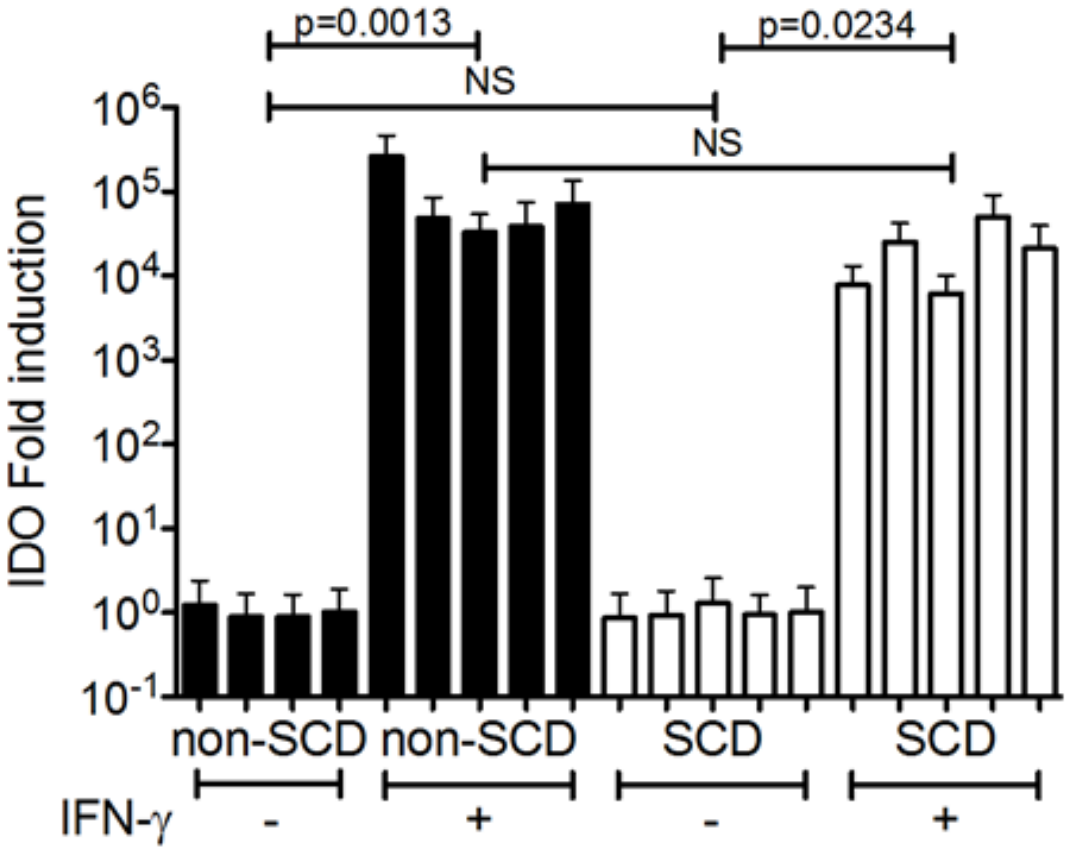


**Figure 5: Flow Cytometry Gating Strategy (representative experiment) for Experiments Assessing Suppression of T cell Proliferation by SCD and non-SCD MSCs**

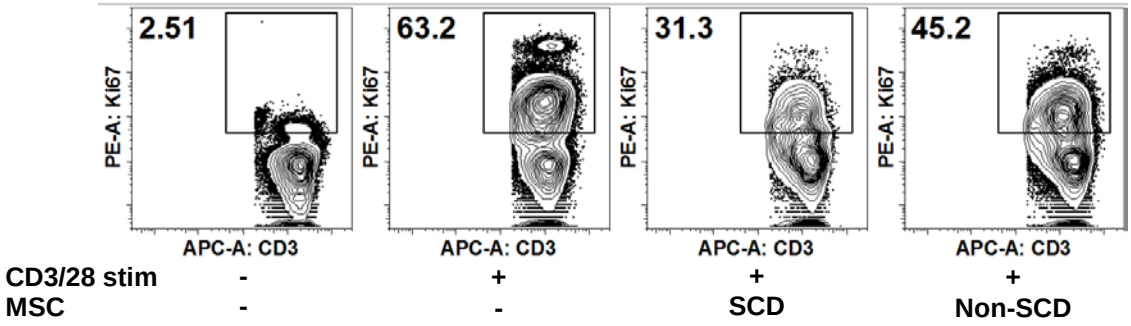


**Figure 6: Suppression of Random Donor T cell Proliferation by Varying Concentrations of SCD (n=5) and non-SCD (n=5) MSCs as Assessed by Flow Cytometry**

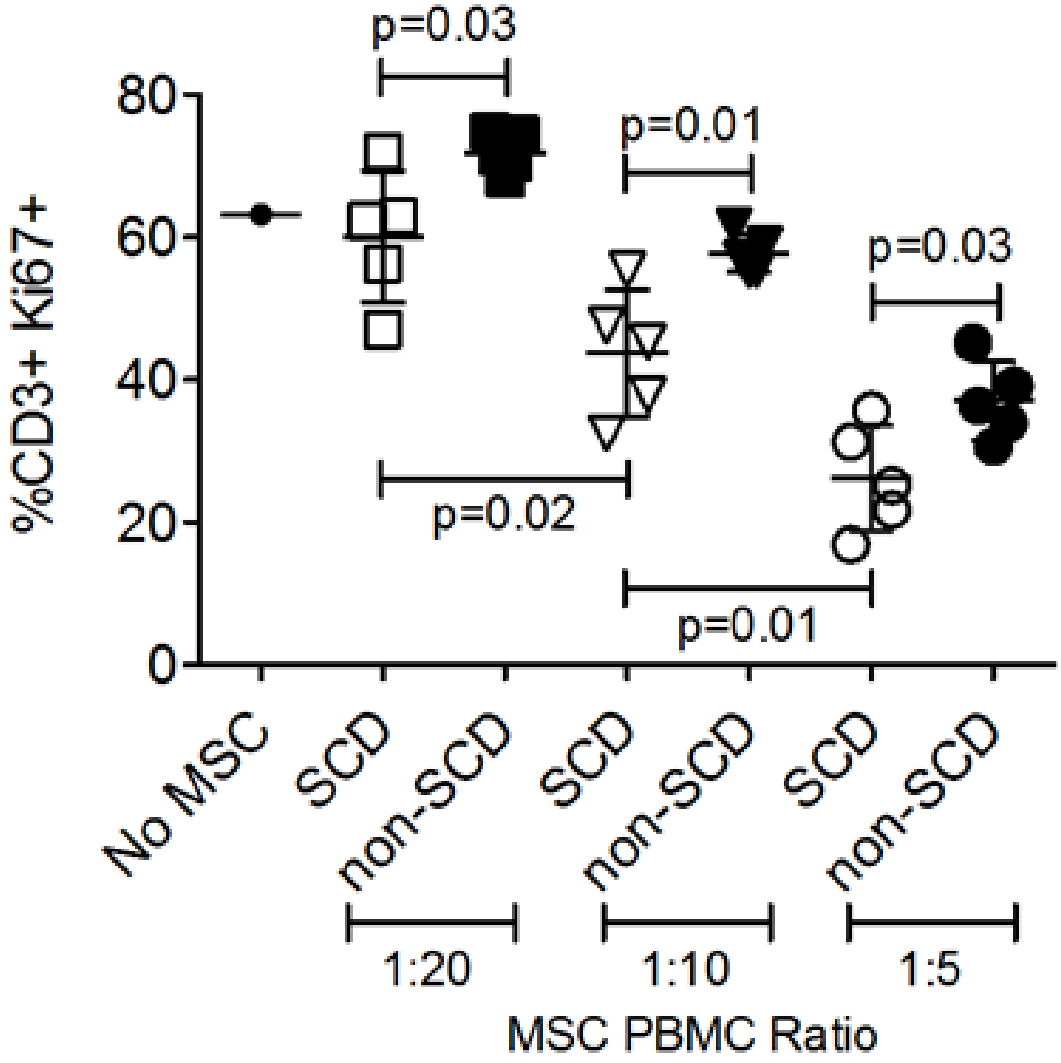


**Figure 7: Suppression of Autologous T cell Proliferation by Varying Concentrations of SCD (n=3) and non-SCD (BMH21) MSCs as Assessed by Flow Cytometry**

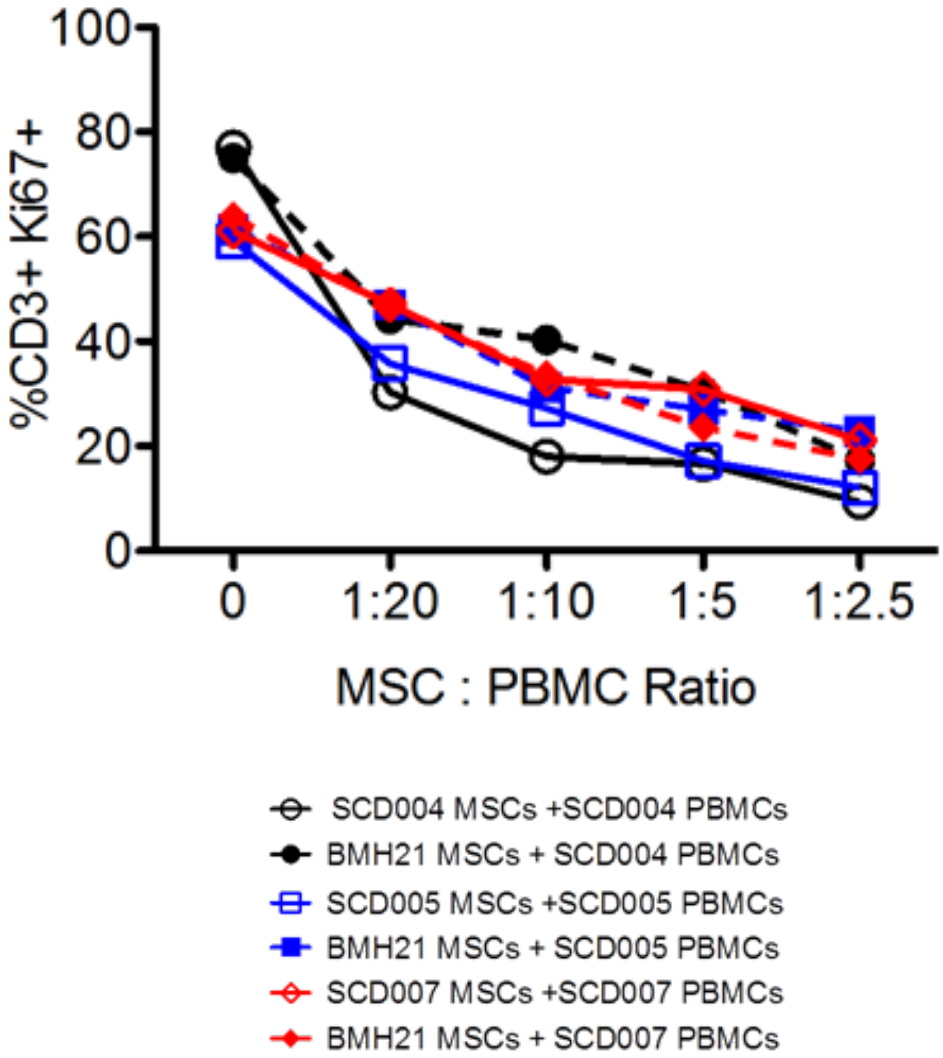


**Figure 8: Heat Map of 48 Hematopoiesis Genes in Unstimulated and IFN-γ Stimulated SCD and non-SCD MSCs (n=5/group) as Assessed by Quantitative PCR using Fluidigm Array**

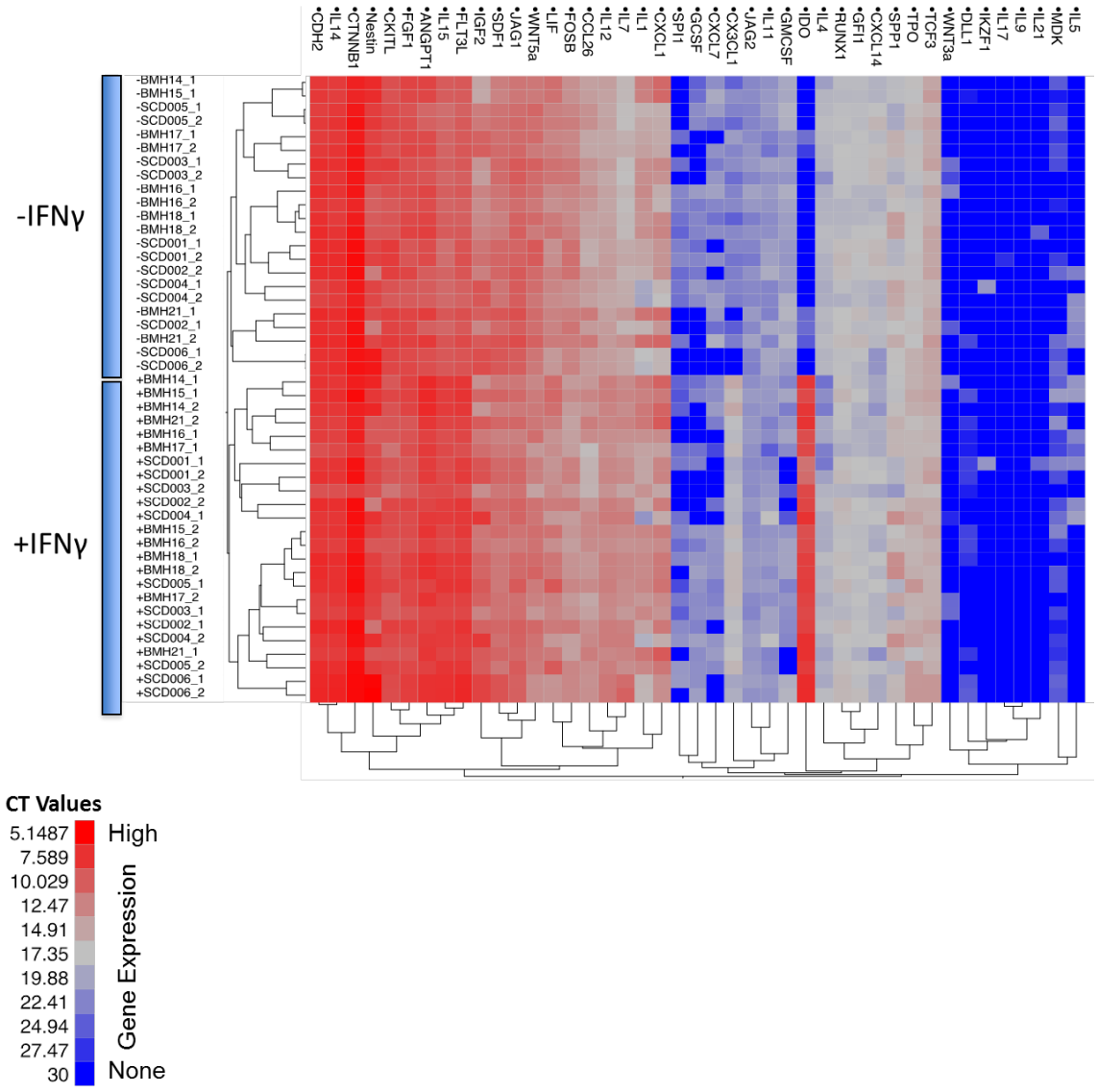


**Figure 9: Volcano Plot of 48 Hematopoiesis Genes Comparing SCD and non-SCD MSCs (n=4-5/group) as Assessed by Quantitative PCR using Fluidigm Array**

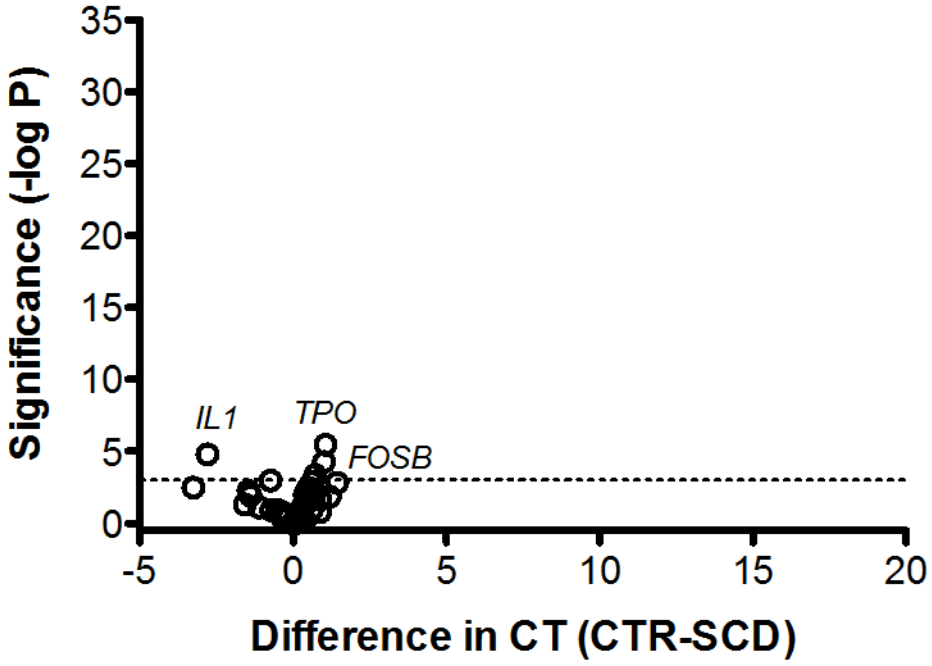


**Figure 10: Volcano Plot of 48 Hematopoiesis Genes Comparing Unstimulated and IFN-γ Stimulated MSCs (SCD and non-SCD, n=4-5/group) as Assessed by Quantitative PCR using Fluidigm Array**

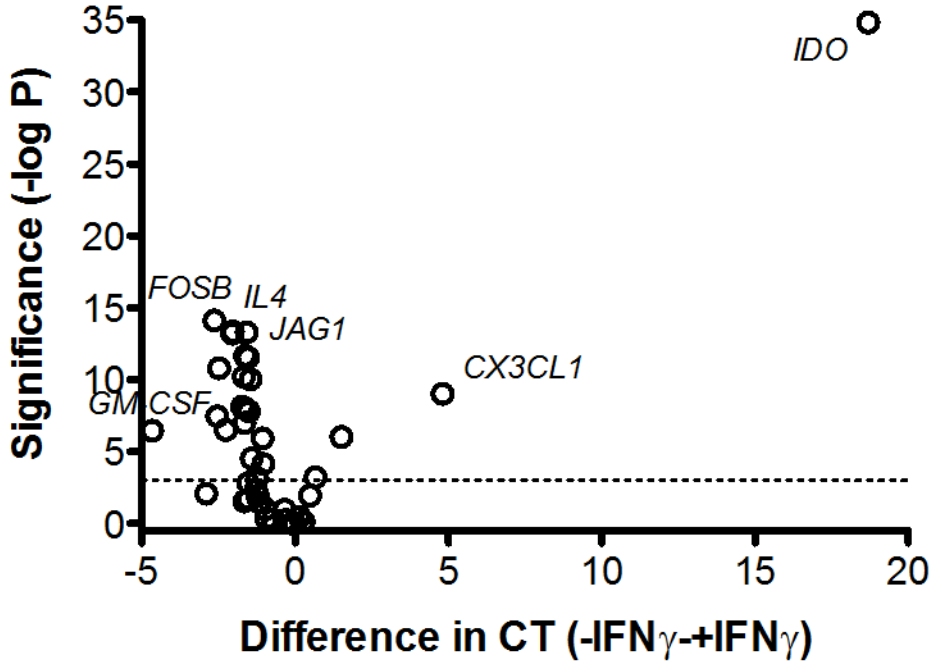


**Table 2: Raw Data of Hematopoiesis Genes that Differ Significantly Between SCD and non-SCD MSCs (n=4-5/group) as Assessed by Quantitative PCR using Fluidigm Array**

| Gene | CT Difference | NLP | P-value |
|---|---|---|---|
| FOSB | 1.008388 | 4.27575071 | 5.29968E-05 |
| IL1 | -2.78854 | 4.8074173 | 1.55805E-05 |
| JAG1 | 0.712404 | 3.36239803 | 0.0004 |
| TPO | 1.056987 | 5.47240299 | 3.36974E-06 |

**Table 3: Raw Data of Hematopoiesis Genes that Differ Significantly Between Unstimulated and Interferon-gamma Stimulated MSCs (n=4-5/group) as Assessed by Quantitative PCR using Fluidigm Array**

| Gene | CT Difference | NLP | P-value |
|---|---|---|---|
| CCL26 | -1.23892 | 3.15976952 | 0.0007 |
| CDH2 | -1.62948 | 11.6723369 | 2.12649E-12 |
| CKITL | -2.02955 | 13.3579676 | 4.38563E-14 |
| CTNNB1 | -1.5364 | 11.5357723 | 2.91224E-12 |
| CX3CL1 | 4.811739 | 9.04176983 | 9.08302E-10 |
| CXCL14 | -2.26259 | 6.52668818 | 2.9738E-07 |
| FGF1 | -1.4525 | 10.0364154 | 9.1957E-11 |
| FOSB | -2.63182 | 14.1270047 | 7.46441E-15 |
| GFI1 | -1.67068 | 10.2195254 | 6.03218E-11 |
| GMCSF | -4.66055 | 6.4666992 | 3.41429E-07 |
| IDO | 18.69824 | 34.8275454 | 1.48749E-35 |
| IL12 | 0.660324 | 3.23052751 | 0.0006 |
| IL14 | -1.58053 | 13.294858 | 5.07157E-14 |
| IL17 | -1.59848 | 7.98759142 | 1.02898E-08 |
| IL4 | -2.53913 | 7.4755111 | 3.34571E-08 |
| IL7 | 1.511584 | 6.02958219 | 9.34153E-07 |
| IL9 | -1.50442 | 7.81812508 | 1.52011E-08 |
| JAG1 | -2.0494 | 13.2047009 | 6.24165E-14 |
| JAG2 | -1.03779 | 4.15813601 | 6.94807E-05 |
| LIF | -1.41347 | 4.54237894 | 2.86828E-05 |
| RUNX1 | -1.05758 | 5.94859018 | 1.12567E-06 |
| SDF1 | -1.65233 | 7.00446896 | 9.89763E-08 |
| TCF3 | -1.72397 | 8.14991581 | 7.08083E-09 |
| WNT5a | -2.48162 | 10.8103578 | 1.54754E-11 |