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Bone Marrow Derived Mesenchymal Stromal Cells from Sickle Cell Disease Patients Display Intact Functionality

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Summary

Hematopoietic cell transplantation (HCT) is the only cure for sickle cell disease (SCD), but engraftment remains challenging in patients lacking matched donors. Infusion of mesenchymal stromal cells (MSCs) at time of HCT may promote hematopoiesis and ameliorate graft-versus-host disease. Experimental murine models suggest MSC major histocompatibility complex compatibility with recipient impacts their *in vivo* function, suggesting autologous MSCs could be superior to third-party MSCs for promoting HCT engraftment. Here we tested whether bone marrow (BM)-derived MSCs from SCD subjects have comparable functionality compared to MSCs from healthy volunteers. SCD MSC doubling time and surface marker phenotype did not differ significantly from non-SCD. Third-party and autologous (SCD) T cell proliferation was suppressed in a dose-dependent manner by all MSCs. SCD MSCs comparably expressed indoleamine 2,3 dioxygenase, which based upon transwell and blocking experiments, appeared to be the dominant immunomodulatory pathway. The expression of key genes involved in hematopoietic stem cell (HSC)/MSC interactions was minimally altered between SCD and non-SCD MSCs. Expression was, however, altered by IFN-γ stimulation, particularly CXCL14, CXCL26, CX3CL1, CKITL, and JAG1, indicating the potential to augment MSC expression by cytokine stimulation. These data demonstrate the feasibility of expanding BM-derived MSCs from SCD patients that phenotypically and functionally do not differ per International Society of Cell

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Therapy essential criteria from non-SCD MSCs, supporting initial evaluation (primarily for safety) of autologous MSCs to enhance haploidentical HSC engraftment in SCD.

Keywords

Mesenchymal stromal cells; sickle cell disease; hematopoietic stem cell transplantation; hematopoiesis

Introduction

Sickle cell disease (SCD) is caused by a β-globin mutation, which leads to recurrent vaso-occlusion and ischemia from poorly deformable RBCs. SCD patients have significant morbidity and early mortality despite complex and expensive medical treatment,[1] with millions worldwide living with SCD,[2] resulting in a significant burden of disease. Hematopoietic cell transplantation (HCT) is currently the only treatment with curative intent, with excellent survival following matched sibling donor transplant,[3] but nearly 70% of patients lack an available HLA-matched donor.[4,5] Early results of HCT for SCD from haploidentical first-degree relatives are promising and suggest that this could substantially expand the donor pool.[6] While haploidentical HCT has been well tolerated by SCD patients, the efficacy of this approach has been limited by high rates of graft rejection. Strategies to diminish this immune-mediated rejection have traditionally relied upon augmentation of pre-transplant conditioning, which leads to more global immunosuppression and risk for infection.

As extensively reviewed,[7,8] mesenchymal stromal cells (MSCs) are rare, multipotent progenitors present in bone marrow (BM) that promote hematopoiesis and have immunoregulatory properties. These properties make MSCs attractive as cellular therapy to modulate the immune system post-HCT. Additionally, MSCs are thought to be less globally immunosuppressive than alternative strategies for augmenting engraftment, which may account for them being well tolerated.[9] When given peri-HCT, MSCs enhance pre-clinical hematopoietic stem cell (HSC) engraftment,[10] and clinical trials have demonstrated promising results.[11,12]

Negative outcomes of graft-versus-host disease (GVHD) trials have led to re-examination of what factors may contribute to MSC function *in vivo*, particularly donor source. Despite the previous belief that MSCs are immunoprivileged, 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.[13] Further, elegant work by Nauta et al[10] demonstrated that MSC donor source significantly impacts their effect on pre-clinical HSC engraftment, wherein recipient-derived MSCs enhance engraftment, donor-derived enhance rejection, and third-party source have no effect. We therefore hypothesize that use of autologous MSCs would provide a mechanistically defined remedy to the limitations of prior attempts at promoting engraftment and additionally may prevent GVHD.

The objective of this study was to verify the ability to *ex vivo* expand functional MSCs from the BM of patients with SCD (as compared to MSCs from healthy volunteers), based upon the mechanistic hypothesis that autologous MSCs could promote haploidentical HSC engraftment through the inhibition of residual recipient T cells and direct support of hematopoiesis.

Methods

MSC expansion

Following IRB approval and informed consent, BM was aspirated from the posterior iliac crest of up to nine healthy adult volunteers (Emory University) and up to eleven pediatric patients with SCD (Aflac Blood and Cancer Disorders Center BMT Program, prior to matched related HCT). MSC culture and isolation occurred as previously described.[14] In brief, BM 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], 100 U/ml penicillin/streptomycin) at 100,000-300,000 cell/cm^2. Non-adherent hematopoietic cells were removed by changing the medium after 3 days of culture and MSCs were allowed to expand for 7-12 days. Thereafter, the cells were passaged weekly by treatment with trypsin/EDTA and reseeded in fresh MSC medium at 1000 cells/cm^2. MSCs were counted at passage 0 (P0) and P1 using an Invitrogen™ Countess™ automated cell counter (Grand Island, NY).

In vitro assays

MSCs underwent flow cytometric analysis for cell surface antigen expression as previously described.[14] In brief, MSCs were cultured for 5-7 days in hPL media, harvested, and resuspended at a concentration of 1×10^6 cells/ml, then analyzed by flow cytometry for the expression of CD45, CD34, CD44, CD73, CD90, CD105, CD19, HLA-I, and HLA-DR (BD BioSciences, San Jose, CA). All samples were run on a Canto II flow cytometer with the appropriate isotype controls. Data is presented as histogram overlay.

RNA from MSCs ±IFN-γ stimulation was extracted and reverse transcribed, and RT-PCR assay was performed for indoleamine 2,3 dioxygenase (IDO) and β-actin, with primers designed using the NCIB/Primer Blast designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; flanking primers IDO_F: 5′TGTAATGCCTACTGAAGAAAC, IDO_R: 5′CTTAAATTATTTTTTGGCTGAATTCAA). Data was then analyzed using the relative quantification method, as previously described.[15] MSCs (±IFN-γ stimulation) and peripheral blood mononuclear cells (PBMCs; from either SCD participants [autologous] or healthy volunteers [third-party] after informed consent on an IRB-approved protocol) were co-cultured as previously described, with proliferation assessed by Ki67 assay according to manufacturer instruction (BD Biosciences, San Jose, CA).[14] Co-culture experiments were repeated (1) with the addition of 1-methyl-DL-tryptophan (1MT; 1 mM; Sigma Aldrich, St Louis, MO) to block IDO and (2) in a transwell system to assess non-contact dependent T cell suppression (Corning Costar 0.4 μM Transwell cell culture inserts, Corning, NY).

Quantitative RT-PCR was performed on MSCs ±IFN-γ stimulation using a Fluidigm 48×48 nanofluidic array[16] targeting forty-seven hematopoiesis genes (plus IDO). Primers were designed to amplify ~20 bp cDNA targets (with product size between 150-200 bp) and were synthesized by Integrated DNA Technologies (Coralville, IA). The targeted genes were pre-amplified in a single 14 cycle PCR reaction after combining cDNA with pooled primers and TaqMan Pre-Amp Mastermix, as described in the manufacturer's protocol (Fluidigm BioMark™, San Francisco, CA). Quantitative amplification of the individual genes (in all samples, with duplication) was subsequently detected using the EvaGreen detection assay on a Biomark I machine and following standard Fluidigm protocols with 30 PCR cycles. Primary data is available online at http://cig.gatech.edu/people/Greg%20Gibson.

Statistical analysis

Data are reported as mean ±SD. Calculations were carried out using GraphPad Prism software (La Jolla, CA). Comparisons between groups were made by two sample t test. Statistical analysis of Fluidigm data was performed using SAS JMP Genomics (Cary, NC) as previously described.[17]

Results

We obtained eleven BM samples from patients with SCD (HbSS genotype; prior to undergoing matched related HCT) ranging 2.6-19.9 years (8.3±4.7) and weighing 12.5-50.8 kg (27.1±11.5). Eight patients received hydroxyurea treatment pre-HCT, which was discontinued approximately two weeks prior to transplant admission. Nine samples were obtained fresh (6-10 ml) and two frozen (2 ml, post-Ficoll), with a starting mononuclear cell (MNC) count of $49.7\pm26.5\times10^6$ in fresh samples. SCD MSCs were expanded from BM harvest to P1 for a total of 14.4±2.5 days.

MSC phenotype was compared between non-SCD and SCD MSCs, with MSCs displaying MSC phenotype ($CD73^+CD90^+CD105^+CD45^-$) consistent with International Society for Cellular Therapy (ISCT) definition (Figure 1A) and with our phase I evaluation of low passage (majority P1) autologous MSCs for Crohn's disease.[18,19] Notably, there was minimal to no $CD45^+$ contamination, consistent with MSCs being the dominant cell at P1. Doubling time (P0 to P1) was calculated and did not significantly differ between SCD and non-SCD samples (Figure 1B). MSCs were then co-cultured with α-CD3/CD28 stimulated PBMCs to evaluate their immunosuppressive function. MSCs suppressed third-party T cell proliferation in a dose- dependent manner, with more potent suppression by SCD MSCs (Figures 1C-D). Given our mechanistic hypothesis, co-culture experiments were repeated using autologous T cells (e.g. MSCs and PBMCs from same SCD donor). As shown in Figure 1E, SCD PBMC proliferation was responsive to autologous MSCs, with comparable suppression by either SCD or non-SCD MSCs.

As IFN-γ-stimulated MSCs have augmented immunomodulatory function upon upregulation of IDO and MSC responsiveness to IFN-γ is a surrogate measure of potency,[21] we next compared IDO gene expression in MSCs ±IFN-γ. Unstimulated MSCs had negligible expression of IDO, whereas IFN-γ stimulation resulted in significant upregulation of IDO by both non-SCD and SCD MSCs (Figure 2A). There was no substantial difference

in IDO expression between non-SCD and SCD MSCs (±IFN-γ). In order to evaluate the contribution of IDO and other contact-independent factors to the suppression of T cell proliferation by SCD MSCs, MSC:PBMC co-culture experiments were repeated in a transwell system. As shown in Figure 2B, MSCs (both non-SCD and SCD) suppressed the proliferation of third-party T cells (%$CD3^+Ki67^+$ cells), consistent with one or more contact-independent pathways being mechanistically dominant. Compared to non-SCD MSCs and as seen in initial experiments, SCD MSCs more potently suppressed T cell proliferation (p=0.0186). Finally, to confirm IDO as the principal pathway for MSC suppression of T cell proliferation, MSC:PBMC co-culture experiments were repeated with a pharmacological blocker of IDO, 1MT. Consistent with blockade of IDO, the addition of 1MT fully abrogated suppression of T cell proliferation by both MSC groups (non-SCD and SCD; Figure 2C-D).

As the soluble factors and surface molecules utilized by MSCs to interact with progenitor HSCs are likely important for MSC promotion of HSC engraftment in HCT, we lastly evaluated the expression of forty-seven such genes (Table 1) in MSCs ±IFN-γ using a Fluidigm qPCR array. In Figure 3A, cycle threshold (CT) for each gene in each MSC sample is depicted as a heat map. Larger CT values indicate lower expression, as lower RNA expression requires more cycles to accumulate detectable product in the RT-PCR reaction. Cluster analysis demonstrated no clustering by MSC donor source (SCD versus non-SCD). In keeping with a MSC pro-hematopoietic effect, twenty genes were highly expressed (red) in all unstimulated MSCs.

Only four genes were significantly differentially expressed in SCD versus non-SCD MSCs, although the volcano plot of significance against CT difference shows that the differential expression in each case was very small (Figure 3B & Table 2). In contrast, twenty-three genes (plus IDO) were significantly differentially expressed in unstimulated versus IFN-γ stimulated MSCs, many with a larger CT difference (Figure 3C & Table 2). These genes included two growth factors, seven cytokines, and four chemokines, in addition to four genes involved in cell fate decisions, one in cell adhesion, and five in cell survival, proliferation, and differentiation.

Discussion

These data demonstrate the feasibility of expanding BM-derived MSCs from patients with SCD prior to HCT with identical growth kinetics, phenotype, and IDO response per ISCT definition[18] to non-SCD MSCs, despite most SCD patients being on hydroxyurea pre-HCT. Importantly, SCD MSCs appear to equally suppress the proliferation of third-party and autologous T cells, the latter of which contribute to graft rejection following haploidentical HCT. Similar to previous mechanistic evaluations of MSC function,[14] IDO appears to be a dominant pathway in the suppression of T cell proliferation by SCD MSCs. In initial hypothesis-generating experiments, we found that SCD MSCs equivalently express (compared to non-SCD MSCs) genes critical for hematopoiesis and highly express twenty of such genes at rest, which may be mechanistically important for MSC augmentation of HSC engraftment.

To our knowledge, these studies are the first to evaluate BM-MSCs from minimally symptomatic pediatric SCD patients, with two studies to date evaluating BM-MSCs from only adults with SCD in the setting of osteonecrosis, where they were found to promote bone repair.[47,48] Additionally, these studies did not evaluate gene expression in SCD MSCs. MSCs have been found to be defective in other BM disorders,[25,49] thus it was crucial to evaluate the phenotype and function of autologous MSCs from this population prior to their clinical use. Given the small number of patient samples evaluated in these studies (including our own) and variability seen between previous studies (including of MSCs from patients with aplastic anemia),[50] additional studies are needed to validate our results.

Compared to previous studies, several notable differences (and similarities) were seen in our evaluation of the gene expression of MSCs. In a previous evaluation of the pro-inflammatory cytokine IL1β in SCD patients, expression was increased in PBMCs relative to healthy controls,[51] while we found decreased expression in the MSCs of our SCD subjects (notably with the largest CT difference). Similar to other inflammatory diseases, polymorphisms of IL1β have been associated with clinical severity of SCD,[52] presumably related to elevated IL1β. The differential expression of IL1β in our study may be due to either our younger, less clinically affected SCD cohort and/or due to inherent differences in IL1β expression in MSCs versus PBMCs.

The expression of several hematopoiesis genes evaluated in our study has also been examined in either healthy individuals or those with other BM disorders. Unlike MSCs from individuals with myelodysplastic syndrome (MDS),[25] IFN-γ stimulated MSCs downregulated CXCL26 and JAG1 and upregulated CX3CL1 compared to unstimulated MSCs; similar to MDS MSCs, expression of CXCL14 and CKITL was decreased. Unlike previous reports of no IL-7 expression by human BM-derived MSCs,[29] we demonstrated expression in both unstimulated and IFN-γ stimulated MSCs, with significant increase in the latter. These studies suggest that expression of hematopoiesis genes may be augmented by IFN-γ stimulation.

Despite the majority of MSC clinical trials ignoring MHC barriers through infusion of largely third-party MSCs, pre-clinical studies suggest that the immunology (e.g. matching) of MSCs is important,[10,13] prompting our goal of using autologous MSCs (where possible) for clinical use. Specifically following allogeneic HCT, clinical studies demonstrate that the bone marrow MSC compartment remains recipient,[53-56] which suggests that the biology of autologous MSCs and resultant immune tolerance is distinct from unmatched third-party or HSC donor-derived MSCs. We therefore hypothesize that MSC donor source significantly contributed to differences in efficacy seen between pre-clinical and clinical trials, particularly in the more extensively studied GVHD setting, although further pre-clinical and clinical studies (including comparing autologous and third-party products) are needed.

Particularly in HCT for SCD and other non-malignant diseases, the peri-HCT infusion of MSCs is attractive for two reasons. First, unlike in HCT for malignant diseases, these patients gain no benefit from GVHD via the graft-versus-tumor effect. As previously reviewed,[57] several small, early phase clinical trials demonstrated an efficacy signal when MSCs were given peri-HCT, with four trials showing no grade III-IV acute GVHD and two

showing no chronic GVHD. Secondly, the risk for graft rejection is higher in these non-malignant diseases, particularly with the use of alternative donor sources. In the case of SCD, high rates of graft rejection following cord blood[58] and haploidentical[6] HCT have limited expansion of this curative therapy to the majority of patients who lack a matched donor option. This was validated in a small pilot trial,[59] where non-HLA-matched MSCs failed to promote cord blood engraftment in two SCD patients following a conditioning regimen similar to the aforementioned cord blood trial,[58] with both patients having primary graft failure with autologous reconstitution. As HSC homing and/or cell dose is likely more important in cord blood transplant, whereas graft failure following haploidentical transplant is predominantly immune-mediated,[60] MSCs could be particularly efficacious in promoting engraftment of haploidentical HSCs. Nonetheless, these studies further highlight the need for caution in the development of trials in high-risk populations and in making efficacy interpretations in small trials underpowered for such.

The primary limitations of these studies are related to the samples, both the small number and the age distribution. BM samples are not routinely obtained from individuals with SCD, and we were able to take advantage of our clinical practice of obtaining a pre-HCT “back-up BM” in our pediatric SCD patients. It is even more difficult to obtain BM samples from healthy pediatric patients, thus our comparison group includes adults only. As the goal of these initial studies of SCD MSCs was to ensure that they met minimal criteria for phenotype and function per ISCT criteria, our samples were adequate for such. Our future phase I clinical trial (detailed below) will include both a larger number of patients and adults with SCD, which will allow us to (1) make the better comparison between adult SCD and non-SCD MSCs and (2) perform more detailed analyses of MSC function. The latter will include a more comprehensive assessment of MSC suppression of T cell proliferation (both through sorting of immune cell subsets and assessment of the role of non-IDO pathways) and of MSC support of hematopoiesis (through MSC:HSC co-culture experiments and validation of our gene expression studies).

In conclusion, our study is the first to evaluate BM-MSCs from minimally affected pediatric SCD patients and to evaluate SCD MSC gene expression, including following cytokine stimulation. As we have validated that SCD MSCs have phenotype and function per essential ISCT criteria, including comparable suppression of autologous T cell proliferation, our next steps include evaluating the safety and early efficacy of autologous MSCs to enhance haploidentical HSC engraftment in patients with severe SCD who lack a matched donor. Additionally, this platform will allow us to more comprehensively evaluate the *in vitro* function of SCD MSCs, including studies recommended by the most recent ISCT position paper.[61]

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Highlights

- SCD MSCs exhibit intact phenotype, replication, and immunosuppressive functionality
- IDO is the dominant immunomodulatory pathway utilized by SCD MSCs
- SCD and control MSCs have comparable expression of genes supporting hematopoiesis

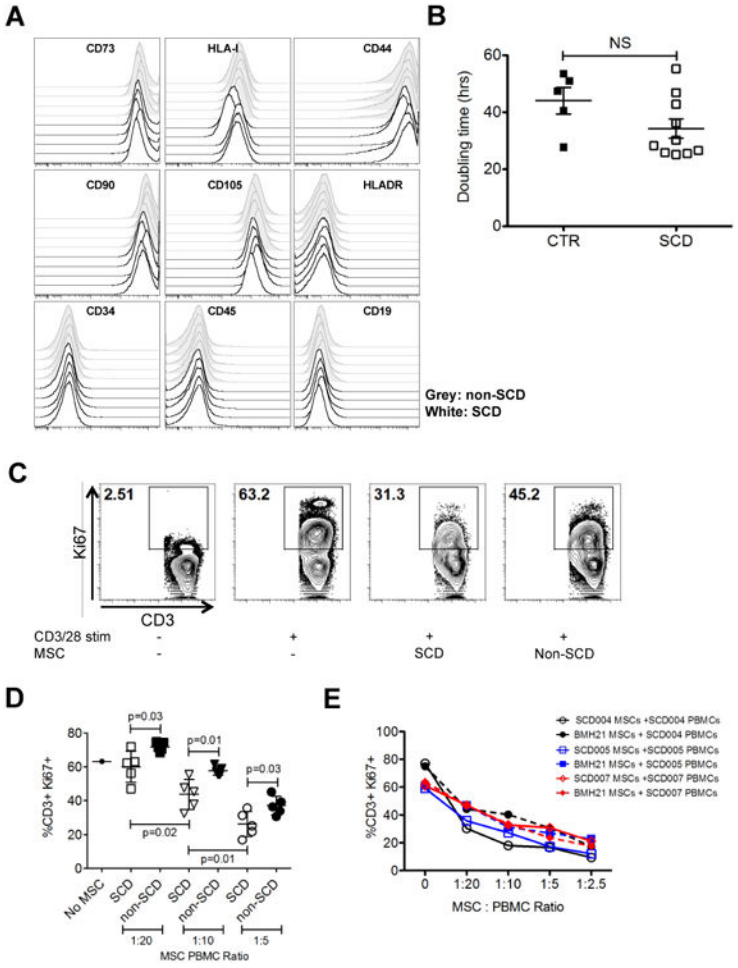

Figure 1. Phenotype and function of SCD and non-SCD MSCs

(A) Low passage (P1-P3) cryopreserved SCD and non-SCD MSCs (n=5/group) were cultured for 5-7 days then analyzed using flow cytometry for the cell surface expression of markers used to identify MSCs. Data presented as histogram overlay of markers (white=SCD, light grey=non-SCD) and unstained samples (dark grey). As per ISCT guidelines,[20] SCD MSCs 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. MFI for each marker was compared between 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 (p>0.05). (B) Growth of SCD MSCs (n=10) was compared to non-SCD MSCs (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). (C) To evaluate the immunomodulatory function of SCD MSCs, MSCs (n=5/group) were co-cultured for 4 days with third-party PBMCs, with or without anti-CD3/CD28 co-stimulation, and T cell proliferation was assessed by flow cytometric analysis of Ki67 expression (%$CD3^+Ki67^+$ cells). Representative flow cytometry gating strategy for a single SCD and non-SCD sample is shown. (D) 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. When PBMCs were co-cultured with MSCs at varying concentrations, both SCD and non-SCD MSCs suppressed T cell proliferation in a dose-dependent manner. SCD MSCs more potently suppressed T cell proliferation at all concentrations compared to non-SCD MSCs. (E) Next, MSCs were 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 again assessed by flow cytometric analysis of Ki67 expression. Results were compared to experiments performed with non-SCD MSCs from a single donor (BMH21). 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 ($p>0.05$ at all MSC:PBMC ratios).

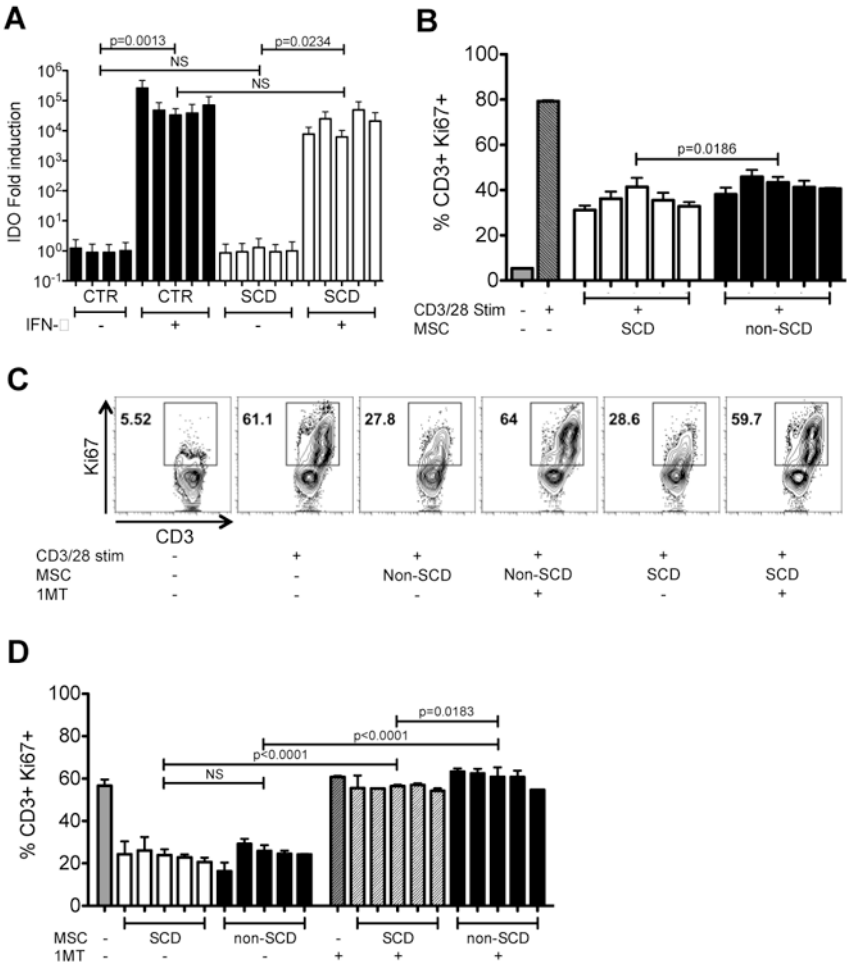

Figure 2. IDO as dominant pathway in suppression of T cell proliferation by SCD and non-SCD MSCs

(A) IDO gene expression was compared 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. 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. There was no difference in IDO expression between non-SCD and SCD MSCs. (B) To evaluate the role of contact-independent pathways (including IDO) in the immunomodulatory function of SCD MSCs, MSCs (n=5/group) were co-cultured in a transwell system for 4 days with third-party PBMCs, with or without anti-CD3/CD28 co-stimulation, and T cell proliferation was assessed by flow cytometric analysis of Ki67 expression (%$CD3^+Ki67^+$ cells). Despite being co-cultured in a transwell system, both SCD and non-SCD MSCs continued to suppress T cell proliferation, with more potent suppression by SCD MSCs. (C) To evaluate the contribution of IDO in this contact-independent suppression of T cell proliferation, co-culture experiments were repeated ±1MT. Representative flow cytometry gating strategy for a single SCD and non-SCD sample is shown. (D) In the presence of 1MT, both SCD and non-SCD MSCs lost their ability

(compared to without 1MT) to suppress T cell proliferation, with proliferation comparable to stimulated T cells without the addition of MSCs.

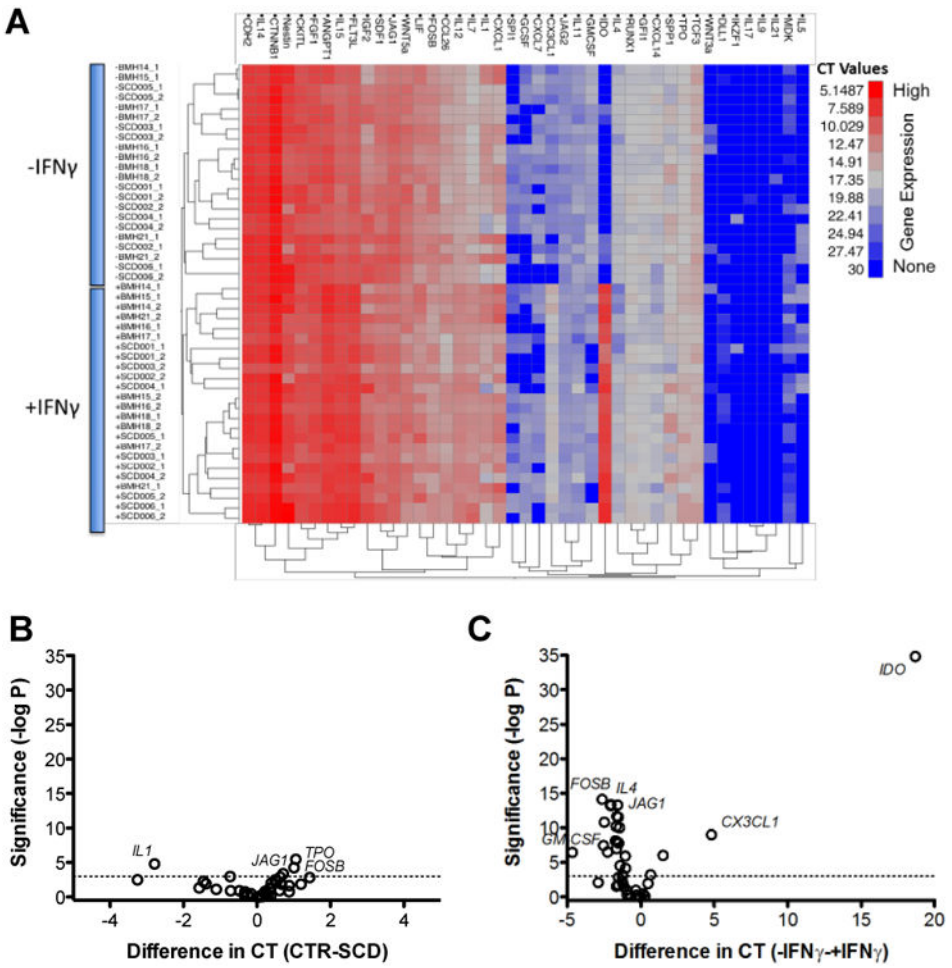

Figure 3. Molecular signature of SCD and non-SCD MSCs in response to IFN-γ

Expression of 47 hematopoiesis genes was assessed using Fluidigm array in unstimulated and IFN-γ stimulated MSCs from individuals with and without SCD (n=4-5/group). (A) Cycle threshold (CT) for each gene in each MSC sample is depicted as a heat map, where red=high expression and blue=low expression. Each sample was duplicated, as indicated by _1 or _2 after the sample ID, where SCD indicates a SCD sample and BMH healthy control MSC. Gene names are indicated across the top of the heat map. Cluster analysis was performed by Ward's method and demonstrated no clustering by MSC donor source (clustering predominantly by IFN-γ stimulation status). (B) Volcano plots contrast the significance (negative logarithm of the p-value, high values more significant) against the difference in average CT value. This plot shows that only four hematopoiesis genes were significantly differentially expressed in SCD versus non-SCD MSCs ($p<0.001$, allowing Bonferroni adjustment for 47 comparisons), although the magnitude of the difference was small (range -2.8 to +1.0). (C) On the contrary, 24 hematopoiesis genes were found to be significantly differentially expressed in unstimulated versus IFN-γ stimulated MSCs ($p<0.001$), most notably IDO, and with a wider magnitude of difference (range -4.7 to +18.7).

Table 1

Molecular Signature of Hematopoiesis Genes in MSCs from Healthy Control and SCD MSCs in response to IFN-γ stimulation

Gene	Gene Name	CT difference (CTR-SCD)	P-value	CT difference (-IFNγ- +IFNγ)	P-value
ANGPT1	Angiopoietin 1	-0.73	0.001	-0.34	0.107
CCL26	C-C motif chemokine ligand 26	-0.25	0.456	-1.24	6.92E-04
CDH2	Cadherin 2	0.19	0.248	-1.63	2.13E-12
CKITL	c-KIT ligand	0.54	0.005	-2.03	4.39E-14
CTNNB1	Catenin beta 1	0.49	0.003	-1.54	2.91E-12
CX3CL1	C-X3-C motif chemokine ligand 1	-1.11	0.077	4.81	9.08E-10
CXCL1	C-X-C motif chemokine ligand 1	-1.46	0.005	-0.07	0.888
CXCL14	C-X-C motif chemokine ligand 14	0.88	0.022	-2.26	2.97E-07
CXCL7	C-X-C motif chemokine ligand 7 (pro-platelet basic protein)	-3.25	0.003	-2.89	0.008
DLL1	Delta-like 1	-0.35	0.448	-0.05	0.906
FGF1	Fibroblast growth factor 1	-0.16	0.345	-1.45	9.20E-11
FLT3L	FMS-like tyrosine kinase 3 ligand	0.22	0.152	0.15	0.338
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1.01	5.30E-05	-2.63	7.46E-15
GCSF	Granulocyte colony stimulating factor	0.07	0.950	-0.10	0.928
GFI1	Growth factor independent 1 transcription repressor	0.35	0.072	-1.67	6.03E-11
GMCSF	Granulocyte-macrophage colony stimulating factor	-1.57	0.048	-4.66	3.41E-07
IDO	Indoleamine 2,3-dioxygenase	-0.72	0.123	18.70	1.49E-35
IGF2	Insulin like growth factor 2	1.20	0.014	-0.28	0.550
IKZF1	IKAROS family zinc finger 1	0.88	0.167	-1.51	0.019
JAG1	Jagged 1	0.71	4.34E-04	-2.05	6.24E-14
JAG2	Jagged 2	-0.33	0.173	-1.04	6.95E-05
LIF	Leukemia inhibitory factor	-0.48	0.121	-1.41	2.87E-05
MDK	Midkine (neurite growth-promoting factor 2)	0.38	0.621	0.28	0.717
Nestin	n/a	0.30	0.526	-1.23	0.011
RUNX1	Runt related transcription factor 1	0.64	0.001	-1.06	1.13E-06
SDF1	Stromal cell-derived factor 1(C-X-C motif chemokine ligand 12)	0.67	0.013	-1.65	9.90E-08
SPI1	Spi-1 proto-oncogene	0.14	0.888	-0.72	0.482

Gene	Gene Name	CT difference (CTR-SCD)	P-value	CT difference (-IFNγ- +IFNγ)	P-value
SPP1	Secreted phosphoprotein 1 (CXXC finger protein 1)	-1.39	0.011	-1.16	0.033
TCF3	Transcription factor 3	0.21	0.375	-1.72	7.08E-09
TPO	Thrombopoietin	1.06	3.37E-06	-0.02	0.910
WNT3a	Wnt family member 3a	0.13	0.855	-1.66	0.028
WNT5a	Wnt family member 5a	0.02	0.947	-2.48	1.55E-11
IL1β	Interleukin 1 alpha	-2.79	1.56E-05	-0.96	0.100
IL4	Interleukin 4	0.62	0.109	-2.54	3.35E-08
IL5	Interleukin 5	0.06	0.959	-0.94	0.425
IL7	Interleukin 7	0.23	0.381	1.51	9.34E-07
IL9	Interleukin 9	0.12	0.581	-1.50	1.52E-08
IL11	Interleukin 11	1.44	0.002	-1.28	0.004
IL12	Interleukin 12	0.35	0.053	0.66	5.88E-04
IL14	Interleukin 14	0.39	0.010	-1.58	5.07E-14
IL15	Interleukin 15	0.00	0.979	0.49	0.011
IL17	Interleukin 17A	0.21	0.347	-1.60	1.03E-08
IL21	Interleukin 21	0.09	0.839	-1.53	0.001

Table 2

Significantly Differentially Expressed Genes and Role in Hematopoiesis and/or Known MSC Expression

Gene	CT Difference	P-value	Role in hematopoiesis and/or known expression by MSCs	Reference(s)
Control versus SCD MSCs				
TPO	1.06	3.37E-06	Stimulates production & differentiation of megakaryocytes, expands mouse HSCs in vitro (w/SCF, IGF-2, and FGF-1), enhances MSC support of HPC proliferation	22,23
FOSB	1.01	5.30E-05	Can reprogram endothelial cells into MPPs (w/GFI1, RUNX1, & SPI1) which engraft into SCID mice	24
JAG1	0.71	4.34E-04	Ligand for Notch 1, overexpressed by MDS MSCs, deletion in endothelial cells reduces number & function of LT-HSCs (steady state & after stress)	25,26
IL1β	-2.79	1.56E-05	Induces production of other cytokines, works in synergy on primitive hematopoietic cells	27
Unstimulated versus IFN-γ stimulated MSCs				
CX3CL1	4.81	9.08E-10	Downregulated in MDS MSC	25
IL7	1.51	9.34E-07	Expressed in nestin+ MSCs (murine), previous report of no expression in human MSCs	28,29
IL12	0.66	5.88E-04	Expressed by MSCs, promotes hematopoiesis (in vivo, in absence of IFN-γ), reduces radiation-induced hematotoxicity (rhesus monkeys)	29-31
GMCSF	-4.66	3.41E-07	Expressed by MSCs	29
FOSB	-2.63	7.46E-15	Can reprogram endothelial cells into MPPs (w/GFI1, RUNX1, & SPI1) which engraft into SCID mice	24
IL4	-2.54	3.35E-08	IL-4 expressing MSCs attenuate murine EAE, stimulates B cell growth	32
WNT5a	-2.48	1.55E-11	Member of WNT pathway, involved in hematopoiesis; increases repopulation of both short- & long-term HSCs through maintenance of HSC quiescence	33,34
CXCL14	-2.26	2.97E-07	Downregulated in MDS MSCs	25
JAG1	-2.05	6.24E-14	Ligand for Notch 1, overexpressed by MDS MSCs, deletion in endothelial cells reduces number & function of LT-HSCs (steady state & after stress)	25,26
CKITL	-2.03	4.39E-14	Expressed by MSCs (including nestin^{+} murine MSCs), enhances MSC support of HPC proliferation, downregulated in MDS MSCs	23,25,28,29
TCF3	-1.72	7.08E-09	Regulates HSC lineage, including self-renewal & lymphoid fate; mice deficient in E2A isoform have HSC exhaustion with LMPP deficiency	35,36
GFI1	-1.67	6.03E-11	Can reprogram endothelial cells into MPPs (w/FOSB, RUNX1, & SPI1) which engraft into SCID mice, mutations associated with congenital & idiopathic neutropenia	24,37
SDF1	-1.65	9.90E-08	Chemokine that chemoattracts MSCs, KO mice have absence of lymphoid & myeloid hematopoiesis (fetal marrow), used in human MSC/HSC interaction in hematopoietic reconstitution (in mice)	38-40
CDH2	-1.63	2.13E-12	Used in human MSC/HSC interaction in hematopoietic reconstitution (in mice)	40
IL17	-1.60	1.03E-08	IL-17R KO mice have impaired hematopoietic recovery s/p radiation, mediates granulopoiesis & causes neutrophilia	41,42
IL14	-1.58	5.07E-14	Expressed by MSCs	29
CTNNB1	-1.54	2.91E-12	Important part of Wnt pathway, which is known to regulate development of hematopoietic tissue	33
IL9	-1.50	1.52E-08	Stimulates the growth of multiple myeloid cell types (including erythropoiesis)	27
FGF1	-1.45	9.20E-11	Expressed by MSCs, enhances MSC support of HPC proliferation	23,43

Gene	CT Difference	P-value	Role in hematopoiesis and/or known expression by MSCs	Reference(s)
LIF	-1.41	2.87E-05	Expressed by MSCs, upregulated in SDF-1 & CXCL7 stimulated MSCs, LIF KO mice have significantly decreased numbers of lin^- $Sca\text{-}1^+$ cells	29,38,44,45
CCL26	-1.24	6.92E-04	Chemokine, upregulated in MDS MSCs & in SDF-1 stimulated MSCs	25,38
RUNX1	-1.06	1.13E-06	Can reprogram endothelial cells into MPPs (w/FOSB, GFI1, & SPI1) which engraft into SCID mice	24
JAG2	-1.04	6.95E-05	Ligand for Notch 2, supports expansion of ST-HSCs	33,46