Cytokines increase engraftment of human acute myeloid leukemia cells in immunocompromised mice but not engraftment of human myelodysplastic syndrome cells

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Supplemental Material and Methods

Hematopoietic cell isolation

Bone marrow cells were isolated from both femurs and tibias of primary recipients after crushing with mortar and pestle and washed until bone chips were white. Cells were treated with ACK buffer to lyse the red blood cells (RBC lysis) and then analyzed for myeloid and lymphoid precursor cells or used further for secondary transplantations For secondary engraftment studies, cells were human-CD45-selected by magnetic beads (Miltenyi Biotec) and the exact enrichment was estimated by flow cytometry. One million human CD45 expressing cells per mouse were passaged into secondary recipient mice, alone or in combination with $5x10^5$ MSC. Engraftment levels were measured by aspiration of the contralateral femur 6 -12 weeks after injections. RBC lysis was not performed when analyzing erythrocyte precursor cells.

Enumeration of Receptor Levels and FISH Analysis

Receptor enumeration for human and CD116 (GM-CSFRα), CD117 (c-kit) and CD123 (IL-R α chain) was measured using the QuantiBRITE PE beads (BD Biosciences) per manufacturer's instructions.

Inv(16) fusion transcript (CBFβ-MYH11) was amplified using RT-PCR and primers CBF-A (GCAGGCAAGGTATATTTGAAGG) and MYH11-B2 (TCCTCTTCTCCTCATTCTGCTC)[1]. Inv(16) FISH was performed using commercial CBFB Break Apart FISH Probe Kit (CytoTest).

Flow Cytometry Analysis

Flow cytometry analysis was performed on a LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA). Bone marrow cells from xenografted mice or primary patient samples were stained with a live/dead marker (LIVE/DEAD Fixable Aqua, Molecular Probes), followed by blocking with CD16/CD32 Fc (BD Biosciences) and staining with antibodies for cell surface markers: CD45-BV711, CD34-PE.CF594, CD123-BV605, CD38-BV786, CD19-BV421, CD45RA-APC.H7 (BD Biosciences); CD33-PE (eBiosciences). Engraftment levels in the xenotransplanted mice are expressed as the percentage of the human CD45 expressing cells among live nucleated cells. Because CD45 expression is gradually lost during erythroid differentiation, the population that expresses CD45 in low levels or is negative for CD45 was selected for further analysis to assess erythroid engraftment. Dead cells

were similarly excluded and cells were again blocked with CD16/CD32 Fc blocking antibody. Cells were then stained with the following antibodies: GlyA-FITC, CD71-APC; CD38-APC, murineCD45-PerCP5.5, Ter119-FITC (eBiosciences); CD45-APC.Cy7 (BioLegend), CD34-PE.Cy7 (BD Biosciences).

Immunohistochemistry

Formalin-fixed decalcified sternums from transplanted mice were paraffin-embedded and sectioned at 5 μm sections. Slides were stained using a Leica Bond III Automated Stainer with Bond Polymer Refine Detection (Leica Biosystems) according to manufacturer's instructions. CD33 ready to use antibody and ER2-20 retrieval were purchased from Leica (Leica Biosystems), whereas Gly-C and PGIIIa were purchased from Agilent (Agilent Technologies, Santa Clara, CA).

Mesenchymal Stem Cells isolation and expansion

Mesenchymal stem cells (MSC) were isolated from either fresh or viably cryopreserved bone marrow mononuclear cells by their ability to adhere to plastic surfaces. In brief, mononuclear cells from patients or normal control bone marrow were cultured overnight in MEM-alpha medium supplemented with 20% FBS, 2 mM L-glutamine and 100 U/mL Penicillin/ 100 μM Streptomycin in standard culture conditions (37 ^{o}C and 5% CO_2). Non-adherent cells were removed, whereas adherent cells were allowed to proliferate and expand. All MSC used *in vivo* were not grown further than passage 4 and were phenotypically characterized as MSC by cell surface marker expression (human MSC Phenotyping kit, Miltenyi Biotec Inc.). MSC are included in the CD45 negative fraction of MNC, do not express any stem or lineage specific markers (CD34, CD20, CD14), and are triple positive for CD73, CD90, and CD105. Their functional ability for trilineage differentiation was tested *in vitro* where the isolated MSC were allowed to differentiate into osteoblasts, adipocytes and chondrocytes as described below.

Mesenchymal Stem Cell differentiation

Ex vivo expanded patient and normal donor-derived mesenchymal stem cells were cultured in cell differentiation medium in order to assess their differentiation potential into osteoblasts, adipocytes and chondrocytes.

Osteoblastic differentiation: MSC were allowed to reach confluence and were further cultured in MEM-alpha supplemented with 20% FBS, 2 mM L-glutamine, 100 U/mL

Penicillin/100 μM Streptomycin, 100 nM Dexamethasone, 10 mM glycerol-phosphate and 50 μM ascorbic acid for a minimum of 3 weeks. Culture medium was replaced with freshly made every 2-3 days. Differentiated cultured cells were fixed with 10% formaldehyde and stained with alizarin red. Extracellular calcium deposits, evidence for the presence of osteoblasts, are stained bright red by alizarin red.

Adipogenic differentiation: MSC were again allowed to reach confluence and then cultured further in adipogenic differentiation medium (MesenCult Human Adipogenic Differentiation Medium, StemCell Technologies, Inc.) according to manufacturer's instructions for 3 weeks. Culture medium was replaced every 2-3 days. Differentiated cells were fixed with 10% formaldehyde and stained with Oil-red O stain. Upon adipocytic differentiation MSC modify their shape and form numerous lipid droplets in their cytoplasm which are colored red when stained with Oil-red O.

Chondrogenic differentiation: To assess chondrocyte differentiation potential, cells were cultured in MSC culture medium. One million MSC were trypsinized and pelleted. Cell pellets were cultured overnight in standard culture conditions, and 24 hours later the medium was replaced by chondrogenic differentiation medium (MesenCult-ACF Chondrogenic Differentiation Medium, StemCells Technologies, Inc.). Cell-pellets were cultured according to manufacturer's instructions for at least 3-4 weeks replacing medium every 2-3 days. Pellets were detached, fixed in 10% formaldehyde, embedded in paraffin, sectioned (5 mm) and stained with Alcian Blue and Fast Red according to standard histopathological procedures. Presence of blue-colored cells is characteristic for chondrocytes.

Construction of lentiviral vectors for *in vivo* imaging

Lentiviral vectors that encode Green Fluorescent Protein (GFP) under the transcriptional control of the EF-1a promoter were generated as previously described[2,3]. Click beetle green (CBG) Luciferase expressing lentiviral vectors in which the CBG sequences were preceded in frame by either an eGFP sequence followed by the 2A ribosomal skipping sequence. These vectors permit dual expression of GFP and CBG luciferase. Lentiviral package PCL USUG PRSV Rev, PGSG pol plasmids were used for pTRPE GFP-CGB lentiviral preparation by transfecting with Lipofectamine 2000 on 293T cells.

Labeling of MSC and *in vivo* imaging

Mesenchymal stem cells were infected with lentiviral particles (containing plasmid: pELNS.CBR-T2A-GFP) designed to express GFP and CBG Luciferase (Luc). In brief, 300,000 MSC were infected overnight by addition of lentiviral particles (MOI = 0.5), in the presence of hexadimethrine bromide (8 μg/mL). Twenty-four hours later the particles were removed and the cells were allowed to grow in regular culture medium for additional 72 hours.

Five hundred thousand MSC-GFP-Luc were intrafemorally injected into busulfan conditioned NSG female mice. MSC trafficking was assessed non-invasively by bioluminescence using the Xenogen IVIS-200 System (Xenogen Spectrum System and Living Image Version 4.3 software, Xenogen Corporation, CA, USA). D-Luciferine was administered intra-peritoneally at 15 mg/g of body weight, 10 min prior to image acquisition. Luminescence counts were measured at 1 min exposure time. MSC-injected mice were followed for as long as needed.

Statistical Analysis

Results are given as mean ± SEM or percentages. For AML engraftments, data reported are mean ± SEM and statistical significance was determined using a two-tailed Student *t* tests.

For MDS studies differences in continuous variables were calculated using either Anova or the nonparametric Mann-Whitney *U* test. Differences with $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed using PRISM (Prism 7 for Mac OS X, GraphPad Software, Inc.).

Supplemental References:

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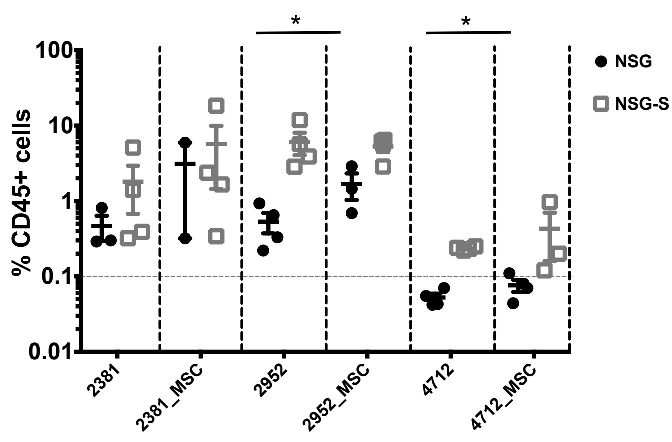
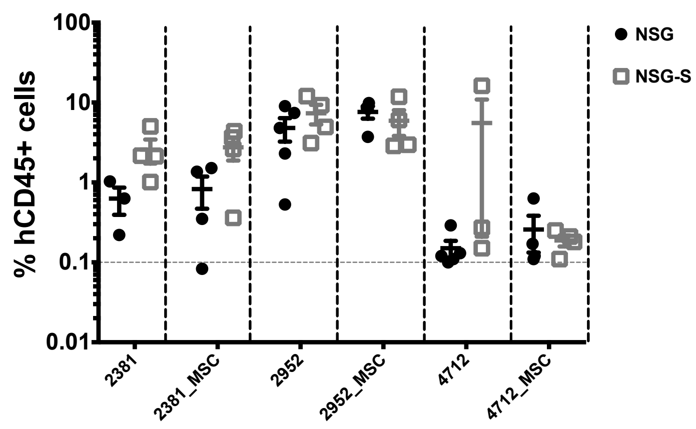
Supplemental Figure 1. MDS engraftment is not impacted by the combination of MSC and human cytokines. Bone marrow MNC alone or with MSC from patients diagnosed with MDS were intrafemorally transplanted into NSG and NSG-S. Percentage of $hCD45^+$ cells in the murine bone marrow was measured at 8 (Supplemental Figure 1A) and 16 (Supplemental Figure 1B) weeks post-transplant.





Supplemental Figure 1