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Supplemental Information

Detailed Characterization of Mesenchymal Stem/Stromal Cells from a Large Cohort of AML Patients Demonstrates a Definitive Link to Treatment Outcomes

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Supplemental Methods

Immunophenotyping of BM-MSC cultures

MSC characterization was performed following the guidelines proposed by the International Society for Cellular Therapy (Dominici et al., 2006). The immunophenotype of cultured BM-MSCs was analyzed by flow cytometry using the following fluorochrome-conjugated monoclonal antibodies: anti-CD73-BV510 (BD. Catalog No. 563198), CD105-FITC (BD. Catalog No. 561443), CD90-APC (BD. Catalog No. 558969), CD13-PerCP-Cy5.5 (BD. Catalog No. 561361), CD45 APC-Cy7 (BD. Catalog No. 560178), CD34 PE (BD. Catalog No. 560941) and CD31-FITC (BD. Catalog No. 560984). Isotype-matched, non-reactive fluorochrome-conjugated antibodies were used as fluorescence controls. Cells (10^6) were incubated for 30 minutes at 4°C in the dark. Data acquisition and analysis was performed using a FACSCanto-II flow cytometer equipped with FACSDiva software (Becton Dickinson) as described (Bueno et al., 2014; Menendez et al., 2009; Rodriguez et al., 2012; Rodriguez et al., 2013).

Adipogenic and osteogenic *in vitro* differentiation of BM-MSC cultures

Differentiation was evaluated by plating BM-MSCs in specific differentiation inductive medium for 2-3 weeks according to manufacturer's instructions (Lonza, Switzerland;(Bueno et al., 2014; Menendez et al., 2009; Rodriguez et al., 2012; Rodriguez et al., 2013). For adipogenic differentiation, cells were cultured in Adipogenic MSC Differentiation BulletKit (Lonza) and differentiated cells were stained with Oil Red-O (Sigma). For osteogenic differentiation, cells were cultured in Osteogenic MSC Differentiation BulletKit (Lonza) and differentiated cells were stained with Alizarin Red-S (Sigma). Differentiation capacity was further quantified by quantitative RT-PCR for adipogenic (C/EBPA, PPAR) and osteogenic (ALPL, Osterix, Osteopontin) specific transcription factors. RNA extraction, cDNA synthesis and PCR conditions have been reported elsewhere (Bueno et al., 2014; Rodriguez et al., 2013).

Proliferative capacity of BM-MSC cultures

Growth kinetics were measured as cumulative population doublings (PD) determined at each passage (Rodriguez et al., 2012; Rodriguez et al., 2013). An equal number of BM-MSCs (1.6×10^3 cells/cm^2) at p1–p5 were initially plated for each patient/donor. Cells were then counted every 9 days and re-plated at 1.6×10^3 cells/cm^2. The number of PDs was calculated by using the formula log10(N)/log10(2), where N represents cells harvested/cells seeded (Conforti et al., 2013).

Clonogenic (CFU-F) assay of BM-MSCs

To assess the clonogenic (self-renewal) capacity of BM-MSCs from HD and patients with AML, cells were seeded in duplicates at P1 at 10 cells/cm^2 in 100-mm dishes. The fibroblast-like colony forming units (CFU-F) clonally emerging from single BM-MSCs were fixed and stained with 1% crystal violet in 100% methanol at day 14 of the assay. Only crystal violet positive (blue) colonies containing more than ~50 cells were scored as CFU-F. The clonogenic potential was calculated as the number of CFU-F counted ×1000/number of cells originally seeded (Mensing et al., 2011).

T-lymphocyte proliferation and cytokine production

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy volunteers by Ficoll-Hypaque gradient centrifugation. Buffy coat units were obtained from the Barcelona Blood and Tissue Bank upon IRB approval. Prior to establishing PBMC:BM-MSC co-cultures, responder PBMCs were labeled with 2.5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions. Then, 1.2×10^5 CFSE-labeled PBMCs were seeded (in triplicate) in 96-well plates in RPMI medium supplemented with 10% FCS, 1% L-glutamine, 0.5% penicillin/streptomycin and 4 mg/ml phytohemagglutinin (PHA) (all from Invitrogen) in the presence of BM-MSCs ($\times10^4$) from HD or LR-, IR-, or HR-AML patients. Negative controls

for immunosuppression were both absence of BM-MSCs and co-culture with transformed/tumoral BM-MSCs (tMSCs), previously reported to lack immunosuppressive potential (Funes et al., 2007; Rodriguez et al., 2014). After 5 days of co-culture at 37ºC in a humidified 5% CO_2 atmosphere, cells were labeled with PerCP-conjugated anti-CD3 (BD. Catalog No. 347344), fixed with 1% paraformaldehyde, and proliferating cells were then determined by CFSE dilution in the $CD3^+$ population. The number of cycling cells was calculated as the % of $CFSE^{mild/low}$ cells that had divided, multiplied by the total number of cells (Gonzalez-Rey et al., 2009; Sanchez et al., 2011).

To determine cytokine production, supernatants from PHA- or lipopolysaccharide (LPS)-stimulated PBMC:BM-MSC co-cultures were collected in triplicate after 5 days and analyzed by Luminex assays (BioRad) for multiplex quantification of IFNγ, IL-1β, TNFα, IL8, IL6 and IL10 (Lopez-Millan et al., 2016). An unstimulated PBMC:BM-MSC co-culture was always processed in parallel as a negative control. For precise cytokine quantification, standard curves were generated using appropriate standards. Standard curves were plotted as cytokine calibrator concentration versus mean PE fluorescence intensity using a four-parameter logistic curve-fitting model (Becton Dickinson, CBA software).

CB collection and $CD34^+$ HSPCs isolation

Umbilical CB units (n=30) from healthy newborns were obtained from the Catalonia Blood Tissue Bank following the institutional guidelines approved by our local IRB. CBs were pooled to reduce variability among individual CB units. The $CD34^+$ cells were purified and assessed as described (Prieto et al., 2016). Only $CD34^+$ fractions with purity >95% were used.

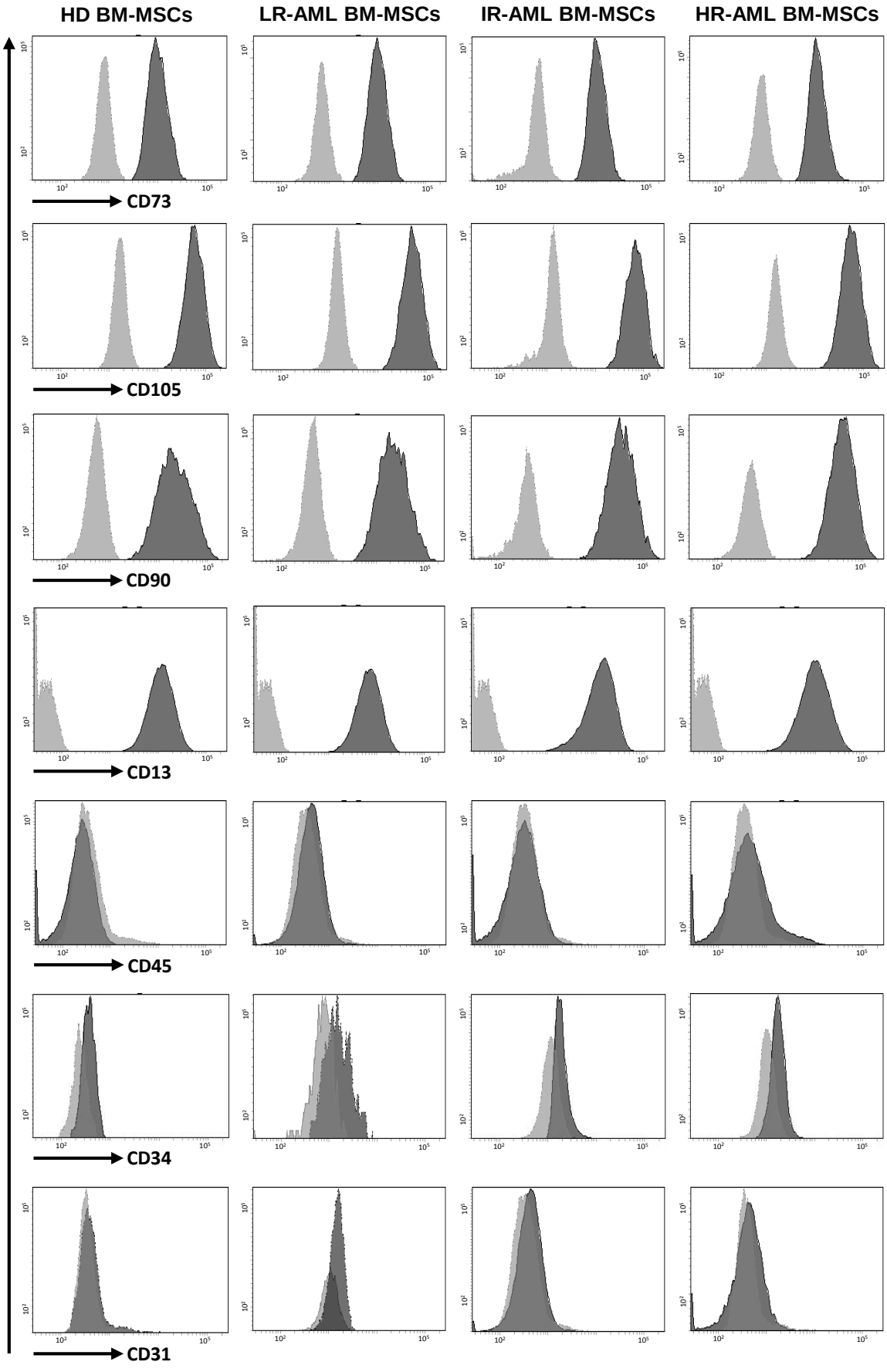
HD BM-MSCs
LR-AML BM-MSCs
IR-AML BM-MSCs
HR-AML BM-MSCs
CD73
CD105
CD90
CD13
CD45
CD34
CD31

Figure Supplementary 1. Immunophenotypic characterization of BM-MSC cultures derived from healthy donors and LR-, IR- and HR-AML patients, analyzed by flow cytometry. Surface markers analyzed were CD73, CD105, CD90, CD13, CD45, CD34 and CD31. Light grey lines represent the control isotypes. Dark gray lines show antibody-specific staining; n=40 patients and n=10 healthy donor controls.