Mesenchymal stromal cells confer chemoresistance to myeloid leukemia blasts through Side Population functionality and ABC transporter activation

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Supplementary material

**Isolation and culture of mesenchymal stromal cells**

Bone marrow mononuclear cells (BM-MNC) were obtained from healthy donors (patient undergoing total hip replacement surgery at the polyclinic of Blois, France). BM-MNCs were counted using an automated cell analyzer (Sysmex) and seeded at 100,000/cm\(^2\) in MEM alpha (Clinisciences) supplemented with 10% fetal bovine serum (FBS) and 10ng/mL of bFGF in order to amplify mesenchymal stromal cells (MSC).

AML MSCs were obtained from sample of bone marrow puncture carried out at diagnosis. AML bone marrow sample was seeded volume to volume in MEM alpha (Clinisciences) supplemented with 10% FBS and 10ng/mL of bFGF.

All MSCs were frozen in MEM alpha medium supplemented with 10% FBS and 10% DMSO (Sigma-Aldrich) for ulterior use.

**Characterization of stromal cell**

Human bone marrow mononuclear cells (BM-MSC) from healthy donors or AML MSCs were cultured in MEM alpha (Clinisciences) supplemented with 10% fetal bovine serum (FBS) and 10ng/mL of bFGF. Medium was replaced after 1 day and then every 3 days, each passage was done at 80% confluence using Trypsin-EDTA (Gibco). Then, MSCs were seeded at 4,000 MSCs/cm\(^2\) for proliferation and 200 MSCs/25cm\(^2\) for clonogenic tests (colony forming unit fibroblast (CFU-F)). Cultures were stopped when the cells were not able to achieve this level of confluence in 21 days. The growth characteristics of MSCs derived from AML patients and healthy donors were compared until the end of proliferation ability by expansion rate calcul.

For CFU-F formation, the culture was stopped by ethanol fixation on day 10 and colored with
Crystal violet (Sigma-Aldrich). All MSCs were frozen in MEM alpha medium supplemented with 10% FBS and 10% DMSO (Sigma-Aldrich).

**Osteogenic, adipogenic and chondrogenic differentiation of MSCs**

For **osteogenic induction**, BM-MSCs and AML-MSCs were plated at 3x10^3 cells/cm^2 in MEMα supplemented with 10% FBS, 0.1µM dexamethasone, 0.05 mM L-ascorbic acid-2-phosphate and 10mM β-glycerophosphate (Sigma-Aldrich, USA) for 21 days of culture. Medium was changed twice a week. Osteogenic cultures were stained histochemically for alkaline phosphatase detection using Abcys detection kit. Matrix mineralization was evaluated by 2% Alizarin Red (AR) (Sigma-Aldrich, USA).

**Adipogenic differentiation** was induced in BM-MSC and AML-MSC subconfluent cultures by 3 treatment cycles with induction media (DMEM supplemented 10% FBS and 1µM dexamethasone, 0.5mM 3-isobuthyl-1-methylxanthine (IBMX), 0.2mM indomethacin and 0.01mg/ml insulin (Sigma-Aldrich, USA)). Cycles were performed during 3-day induction culture and were followed by 1-3 days of maintenance culture in a maintenance medium (DMEM supplemented 10% FBS and 0.01mg/ml insulin) until day10. Between day 10 and day 21, cells were cultivated in maintenance medium refreshed twice a week. Adipogenic monolayer cultures were then histochemically stained with oil red O allowing lipid droplet detection (Cayman chemical, USA).

For **chondrogenic induction**, BM-MSCs and AML-MSCs were centrifuged at 500g for 5min without brake to form small pellets and cultured for 21 days in DMEM supplemented 10% FBS, 1mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 10⁻⁷M dexamethasone and 10ng/mL recombinant TGF-b3. After 3 weeks, cell pellets were resuspended with a graded series of ethanol treatment prior to being embedded in paraffin. Paraffin sections of 5 µm thickness were deparaffinized and stained with Alcian Blue.
Senescence assay

MSC cultures from primary AML cells and healthy donors were stained for β-galactosidase using senescence cells histochemical staining kit (Sigma-Aldrich) according to the manufacturer’s specifications. Senescent stained cells were counted on photography using the ImageJ software.

Immunophenotyping of MSCs

Specific surface antigen expression was realized to characterize MSCs. MSCs were stained with anti-CD45 (clone J.33), anti-CD90 (clone F15-42-1-5), anti-CD105 (clone 1G2) anti-CD73 (clone AD2) (all from Beckman Coulter) and were analyzed using BD Fortessa apparatus (Beckon Dickinson) with Diva Software.

Transwell and neutralization experiments

For transwell experiments, AML blasts were cultivated in the upper chamber of a 3µm pore transwell laid on confluent HD MSCs.

For neutralization experiments, primary AML cells were incubated in SynH with anti-CD49d (α4), anti-CD44, anti-CD29 (β1) antibodies or their control immunoglobulins (Table S1). For inhibition of signal transduction pathways, inhibitors or vehicle controls were added to blast-MSC co-cultures (Table S1).

Table S1: Neutralizing antibody and inhibitor references and characteristics

<table>
<thead>
<tr>
<th>Antibody/Inhibitor</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD44</td>
<td>Progen</td>
<td>DF1485</td>
<td>1</td>
</tr>
<tr>
<td>Anti-CD49d (α4)</td>
<td>R&amp;DSystems</td>
<td>2B4</td>
<td>20</td>
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<tr>
<td>Anti-CD29 (β1)</td>
<td>BD Biosciences</td>
<td>Mab13</td>
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</tr>
<tr>
<td>Control IgG1</td>
<td>BD Biosciences</td>
<td></td>
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</tr>
<tr>
<td>Control IgG2a</td>
<td>BD Biosciences</td>
<td>R-3595</td>
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<tr>
<td>Molecule</td>
<td>Pathway</td>
<td>Manufacturer</td>
<td>Concentration</td>
</tr>
<tr>
<td>------------</td>
<td>---------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>SRC</td>
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<td>100nM</td>
</tr>
<tr>
<td>LY294002</td>
<td>AKT</td>
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<td>10nM</td>
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<tr>
<td>CAS2859866314</td>
<td>Stat5</td>
<td>Millipore</td>
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</tr>
<tr>
<td>LY2090314</td>
<td>GSK3β</td>
<td>Sigma</td>
<td>1nM</td>
</tr>
</tbody>
</table>

**SP cell detection and characterization**

Hoechst staining was performed as previously described\(^1\_2\). Briefly, \(10^6\) cells/ml were suspended in prewarmed (37° C) Dulbecco’s modified Eagle’s medium containing 2% FCS / 10mM HEPES / Hoechst 33 342 (final concentration: 5µg/10^6 cells/ml) and incubated at 37°C for 90 min.

*Cell cycle analysis:* Pyronine Y (50ng/ml; Sigma Aldrich) was added to the cell suspension during the last 15 min of Hoechst staining.

**Transcriptomic analysis**

After co-cultures and Hoechst staining, SP cells were sorted using FACSaria III SROP (BD Biosciences). Total RNAs were extracted using RNeasy microkit (Qiagen). Quantification of the RNA was performed on NanoDrop and its quality was assessed on Bio-analyzer 2100 (Agilent Technologies, CA). Transcriptome probes were synthetized starting with nucleic acid obtained from samples with RIN over 7, low quantity linear amplification was performed by following manufacturer instructions (Affymetrix, CA). Labeled probes were hybridized on Affymetrix HumanGene2.0ST microarray and scanned on Affymetrix station (Genom’IC, Cochin Institute facility). Microarray CEL files were normalized with Expression Console version 1.3 by RMA method (Affymetrix, CA). Gene set enrichment analysis (GSEA) was made with GSEA software version 2.2.0 with MSigDb database version 6.0\(^3\). Raw
transcriptome data were deposited on Gene Expression Omnibus (GEO) academic data repository under the access number GSE114633.

**Patient-derived xenograft (PDX) model**

Animals were used in accordance to a protocol reviewed and approved by the French Institutional Animal Care and Use (Committee of “Midi-Pyrénées” region-France). NOD/LtSz-scid/IL-2Rychainnull (NSG) mice were produced at the Genotoul Anexplo platform of Toulouse (France) using breeders from Charles River Laboratory. NSG mice (6–9 weeks old) were sublethally treated with busulfan (30 mg/kg/day) 24 hours before intravenous injection of $1 \times 10^6$ leukemia cells in 200 µL of Hank's Balanced Salt Solution. Transplanted mice were treated with antibiotic (Baytril) for the duration of the experiment. Eight to 18 weeks after AML cell transplantation and when mice were engrafted (tested by flow cytometry on PB or BM aspirates), NSG mice were treated by daily intraperitoneal injection of either cytarabine (30 mg/kg; kindly provided by the pharmacy of the Toulouse University Hospital) or PBS for control mice, for 5 days. At the end of the 5 days, mice were killed and presence of SP cells was analyzed in the BM as described above.

**References**


Table S2: Combination used to study ABC transporter functionality

<table>
<thead>
<tr>
<th>MDR pump</th>
<th>ABCB1</th>
<th>ABCC1</th>
<th>ABCG2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probes (Ex/Em)</strong></td>
<td>Dioc₂(3) (488/530)</td>
<td>CMFDA (488/530)</td>
<td>Purpurin 18</td>
</tr>
<tr>
<td><strong>Provider</strong></td>
<td>Molecular probes</td>
<td>Molecular probes</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td><strong>Concentration</strong></td>
<td>5ng/mL</td>
<td>0,2µM</td>
<td>30µM</td>
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<td><strong>Viability marker</strong></td>
<td>Sytox red</td>
<td>Sytox red</td>
<td>Iodure de propidium</td>
</tr>
<tr>
<td><strong>CD45 (clone HI30, Sony)</strong></td>
<td>CD45 APC-Cy7</td>
<td>CD45 BV421</td>
<td>CD45 FITC</td>
</tr>
</tbody>
</table>
Figure S1

Patient information's Graph (A) represents the distribution of AML subtypes used in the study. Table (B) gathers AML sample characteristics including the blast percentage evaluated at diagnosis by senior hemopathologists who reviewed each blood smear.

### Table (B)

Gathers AML sample characteristics including the blast percentage evaluated at diagnosis by senior hemopathologists who reviewed each blood smear.
Figure S2: MSCs from AML patients exhibit a decreased expansion capacity and clonogenicity related to senescence

Graph A shows the expansion capacity of MSCs from HDs (black, n=5) or AML patients (red, n=7). The Cumulative number of population doubling for each kind of MSCs is represented per the cumulating number of days between passages. Graph B shows the clonogenicity of MSCs from HDs (black, n=5) or AML patients (red, n=7). The CFU-F number for 200 plated MSCs is represented for each cell passage. MSCs from AML patients exhibit a reduce clonogenicity compared to MSCs from HDs (p<0.001, n=5-8, Wilcoxon test). Graph C shows an increase of β-galactosidase+ MSCs from AML patients (red, n=10) compared to MSCs from HD (black, n=7) at passage 4 (p=0.018 with Wilcoxon test).
**Figure S3**

**A**

Counts

![Histograms for CD90, CD73, and CD105](image)

**B**

Non-induced HD MSCs | HD MSCs | AML MSCs

- Osteoblasts (Alizarin red)
- Adipocytes (Oil red O)
- Chondrocytes (Alcian Blue)

**C**

Isotype

HD MSCs: n=8
AML MSCs: n=5

![Alizarin red quantification](image)

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**Figure S3: Characterization of MSCs from AML patients**

Histograms A show FACS analyses of BM MSCs from HDs patients (black) and from AML patients (red) for CD90 and CD73 and CD105 antigens. MSCs from HDs or from AML patients were differentiated into osteoblasts, adipocytes and chondrocytes. Osteoblastogenesis was evaluated (B) and quantified (C) by alizarin red staining, adipogenesis was evaluated by oil red O staining (B) and chondrogenesis was evaluated by Alcian blue staining (B).
Figure S4: Analysis of Dioc2,3, Purpurin 18, CMFDA efflux by AML blasts patient per patient

Histogram shows the percentage of AML blasts that efflux specific probes (Dioc2,3, Purpurin 18, CMFDA for ABCB1, ABCG2, ABCC1, respectively), patient per patient, after a 3-day culture with or without MSCs from HDs.
Figure S5: Transcriptomic analysis of ABC transporters in SP and MP from AML patients
Heatmap represents the expression level of ABC transporters obtained by transcriptomic analysis.
Figure S6: Quantification of CD123⁺ cells in SP and MP blast population
Graph shows the percentage of CD123⁺ blasts within the CD34⁺ CD38⁻ SP or CD34⁺ CD38⁻ MP populations (p=0.03 with paired t test, n=3).