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 differenciation 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^{-7}M 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

Antibody/Inhibitor	Manufacturer	Clone	Concentration (µg/ml)
Anti-CD44	Progen	DF1485	1
Anti-CD49d (α4)	R&DSystems	2B4	20
Anti-CD29 (β1)	BD Biosciences	Mab13	1
Control IgG1	BD Biosciences		20
Control IgG2a	BD Biosciences	R-3595	1

Molecule	Pathway	Manufacturer	Concentration
Dasatinib	SRC	Cell Signaling	100nM
LY294002	AKT	Cell Signaling	10nM
CAS2859866314	Stat5	Millipore	50 µM
LY2090314	GSK3β	Sigma	1nM

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 SORP (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-2Rγchain^{null} (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 sub-lethally treated with busulfan (30 mg/kg/day) 24 hours before intravenous injection of 1-10 × 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

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Table S2: Combination used to study ABC transporter functionality

MDR pump	ABCB1	ABCC1	ABCG2
Probes (Ex/Em)	$Dioc_2(3)$ (488/530)	CMFDA (488/530)	Purpurin 18
Provider	Molecular probes	Molecular probes	Santa Cruz
Concentration	5ng/mL	0,2µM	30µM
Viability marker	Sytox red	Sytox red	Iodure de propidium
CD45 (clone HI30, Sony)	CD45 APC-Cy7	CD45 BV421	CD45 FITC

Figure S1

A

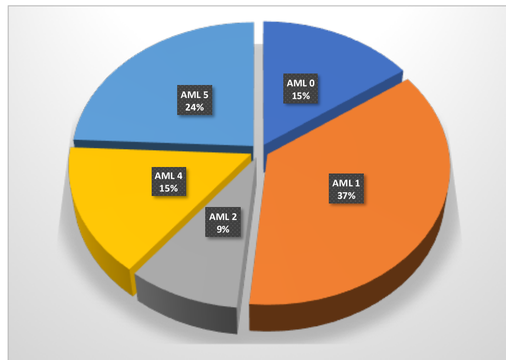

Figure S1: Patient information's

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.

B

Patient number	Hospital	Gender	age	WHO	FAB	% of circulating blasts	Cytogenetic group	Karyotype	Molecular biology	Treatment	Evolution	Death
P1	Percy	F	26	aml with inv(16)	AML4Eo	30% - 41 %	fav	inv 16	rearrangement CBFbeta/MYH1	Daunorubicin + Aracytin	2 CR, 2 relapse	yes
P3	Percy	M	79	aml NOS	AML0	71%	unfab	monosomy 7 , Y deletion	not realized	Daunorubicin + aracytin, VIDAZA and then Amsacrin + Aracytin	1 CR, 1 relapse	yes
P5	Percy	M	60	aml NOS	AML5B	92%	int	monosomy 16	NPM1 non mut, FLT3 non mut	Idarubicin+ Aracytin (failure) then Azacitidin+ Gemtuzumab	1 CR, 1 relapse	yes
P6	Percy	F	56	aml with mutated NPM1	LAM4	27%	int	normal	NPM1 mut, WT1 over expressed	Daunorubicin + Aracytin puis Daunorubicin+ aracytin + mylotarg	1 CR, molecular relapse, CR2 and graft	no
P7	Percy	F	79	aml NOS	AML1	80%	unfav	complex monosomy 15 and 17	not realized	Daunorubicin + aracytin (failure) then Azacitidin	relapse	unknown
P10	Percy	M	75	aml NOS	AML1	18%	unknown	normal	not realized	Aracytin	CR	no
P12	Percy	M	45	aml NOS	AML2	15%	Int	normal	WT1 over expressed	Daunorubicin+ aracytin then mylotarg after 1st graft failure	graft, CR, relapse, graft, no remission	yes
P16	Percy	M	36	aml with biallelic mutation of CEBPA	AML1	65-90%	int	normal	WT1 over expressed CEBP alpha mut	daunorubicin + aracytin	CR	no
P17	Percy	M	65	aml with t(16;16)	AML4Eo	43%	fav	Inv 16	MYH11/CBFp +	Daunorubicin + aracytin (failure) then Aracytin + Mylotarg	relapse	yes
P19	Percy	M	67	aml with mutated NPM1	AML1	98%	int	trisomy 8	NPM1 mut , WT1 over expressed, tandem duplication Flt3	Daunorubicin + cytarabin	shock after treatment	yes
P21	Percy	M	76	aml with mutated NPM1	AML1	95%	int	normal	tandem duplication FLT3 , NPM1 mut	Daunorubicin + aracytin	CR	no
P23	Percy	M	62	aml NOS	AML5A	85%	int	Trisomy 8	FLT3 mut, WT1 over expressed	Daunorubicin + Aracytin(failure) then mylotarg + cytarabin	CR, relapse, CR, graft	no
P26	Percy	M	64	aml with mutated NPM1	AML1	82%	unfav	Trisomy 11	NPM1+ et WT1 over expressed +FLT3 mut, MLL	Daunorubicin + aracytin	graft, CR	no
P27	Percy	M	70	aml NOS	AML0	95%	int	Trisomy 13	not realized	Aracytin + Idarubicin	relapse	Yes
P28	Percy	F	76	aml NOS	AML5	90%	Int	normal	not realized	LD aracytin	refractory relapse	yes
P33	Percy	F	37	aml NOS	AML2	14%	Int	normal	wt1 over expressed	Daunorubicin + cytarabin then Aracytin	CR	no
P39	Percy	M	66	aml NOS	AML5b	75-85%	Int	normal	normal	Aracytin + Idarubicin	refractory relapse	yes
P41	Percy	F	75	aml NOS	AML5A	40%	Normal	normal	not realized	Vidaza	relapse	unknown
P42	Percy	F	34	aml with biallelic mutation of CEBPA	AML2	76%	fav	normal	CEBPa duble mut	3+7 Daunorubicin	unknown	yes
P44	Percy	F	53	aml with mutated NPM1	AML1	86%	int	normal	NPM1 mut FLT3 mut	3+7 idarubicin	relapse	Yes
P47	Percy	F	53	aml NOS	AML1	87%	unfav	deletion of 7	normal	3+7 idarubicin	relapse	yes
P48	Percy	M	66	aml with t(11;X)	AML0	31%	unfav	trisomy 18 t(11;X)	MLL	3+7 idarubicin	CR	no
P51	Percy	M	48	aml with mutated NPM1	AML5A	52%	int	normal	NPM1 mut FLT3 itd	3+7 daunorubicin	relapse	unknown
P52	Percy	M	74	aml with mutated NPM1	AML5A	53%	Int	inv 9 (constit)	NPM1 mut	3+7 idarubicin	CR	no
P53	Percy	M	50	aml with mutated NPM1	AML5A	77%	Int	normal	NPM1 mut	3+7 daunorubicin	CR	no
P57	Percy	F	57	aml with mutated NPM1	AML1	81%	int	X deletion	NPM1 mut FLT3 itd	3+7 idarubicin	CR	no
P61	Percy	M	61		AML1	53%	Int	normal	CEBPa simple mut	3+7 idarubicin	CR	no
P62	Percy	F	44	aml with mutated NPM1	AML5	54%	fav	normal	NPM1 mut	3+7 daunorubicin	CR	no
P67	Percy	M	79	aml with inv(16)	AML4	29%	fav	inv 16	not realized	Vidaza		
P55	St louis	M	89	aml NOS	AML1							
P56	St louis	F	44	aml NOS	AML4							yes
P58	St louis	F	24	aml NOS	AML0 bi		nc	complex, hyperdiploidy	del NR3C1, del CREBBP, del CDNK2a	3+7 daunorubicin + vindesin-dexa	CR	no
P59	St louis	M	57	aml with t(16;16)	AML4Eo		fav	inv16	CBFbeta-MYH11	3+7 daunorubicin	Death before CR (septic shock)	yes
P60	St louis	F	56	aml with mutated NPM1	AML1		fav	normal	NPM1 mut	3+7+Mylotarg	CR	no

Figure S2

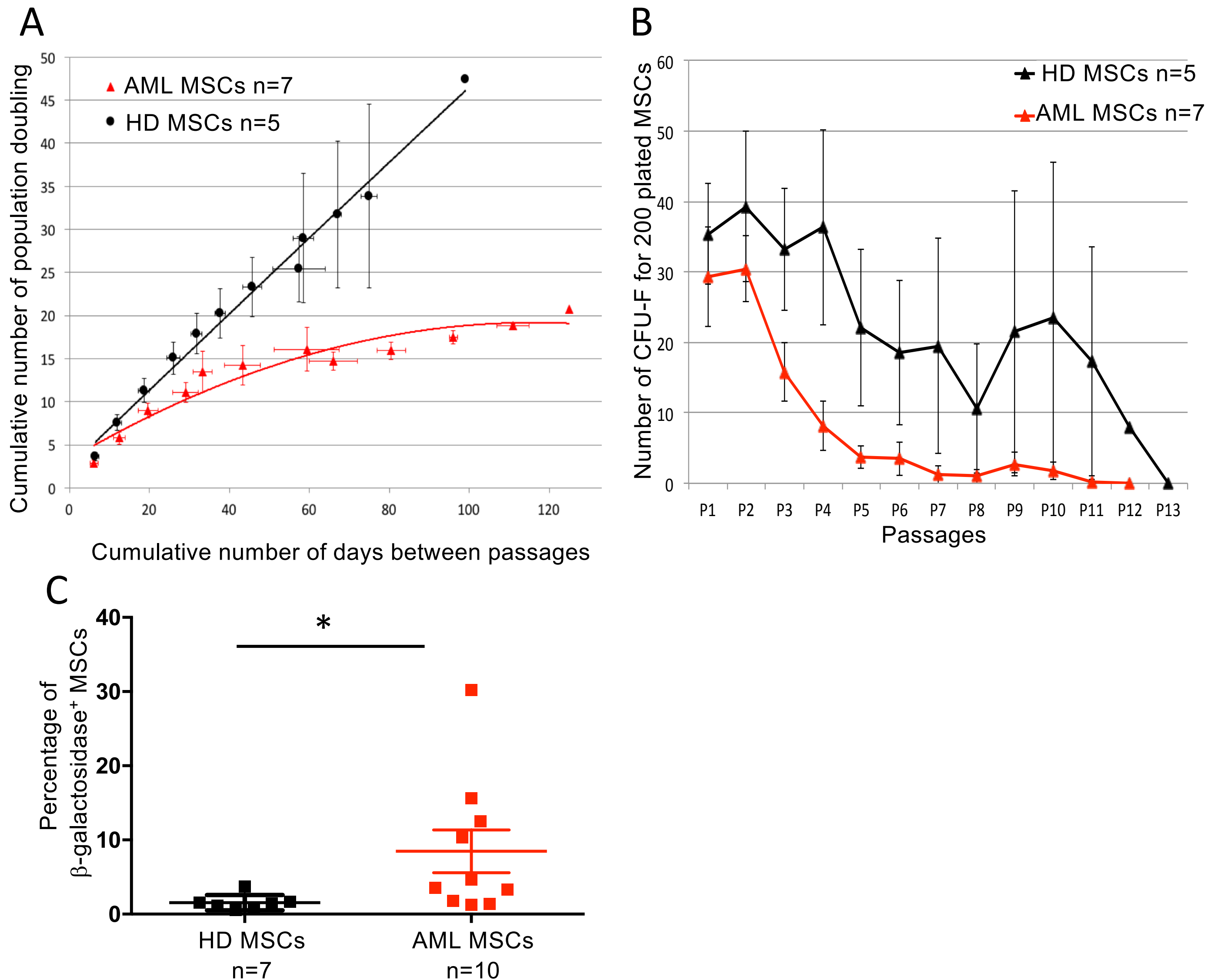

Figure S2: MSCs from AML patients exhibit a decreased expansion capacity and clonogenicity related to senescence

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).

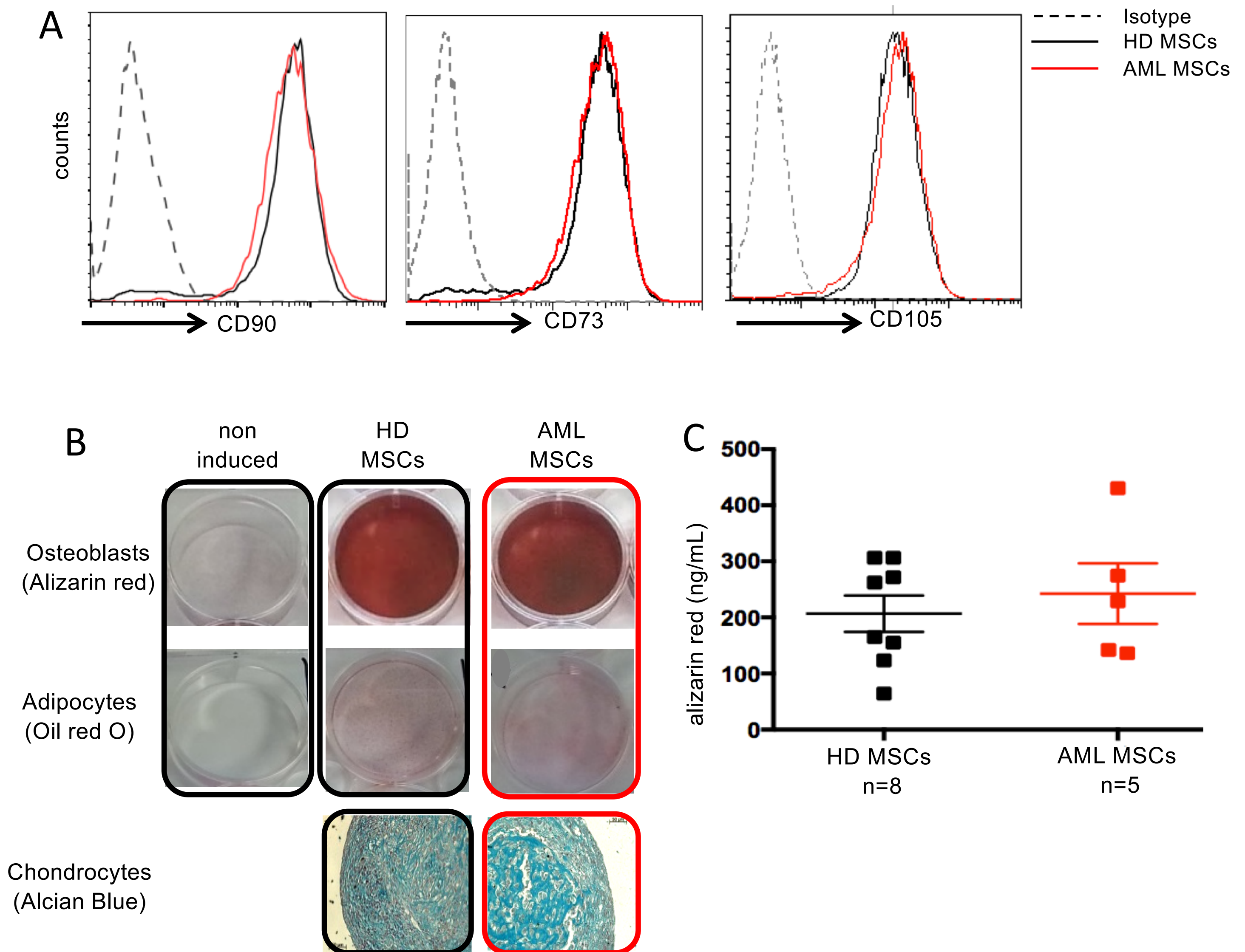

Figure S3: Characterization of MSCs from AML patients

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, adipogenenesis was evaluated by oil red O staining (**B**) and chondrogenesis was evaluated by Alcian blue staining (**B**).

Figure S4

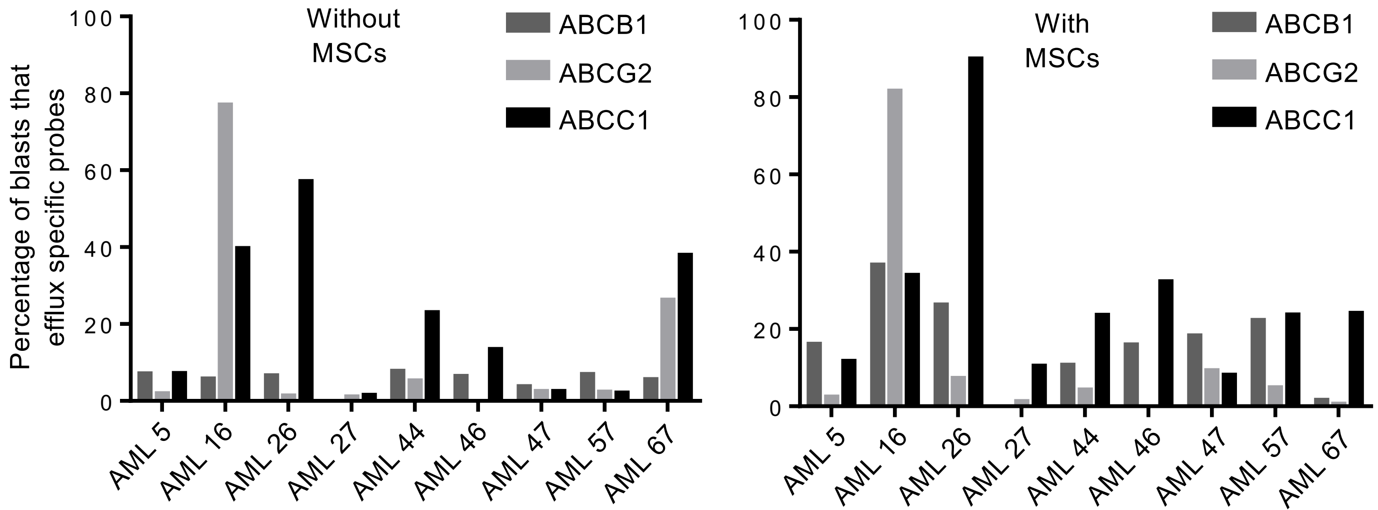

Figure S4: Analysis of Dioc2,3, Purpurin 18, CMFDA efflux by AML blasts patient per patient

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

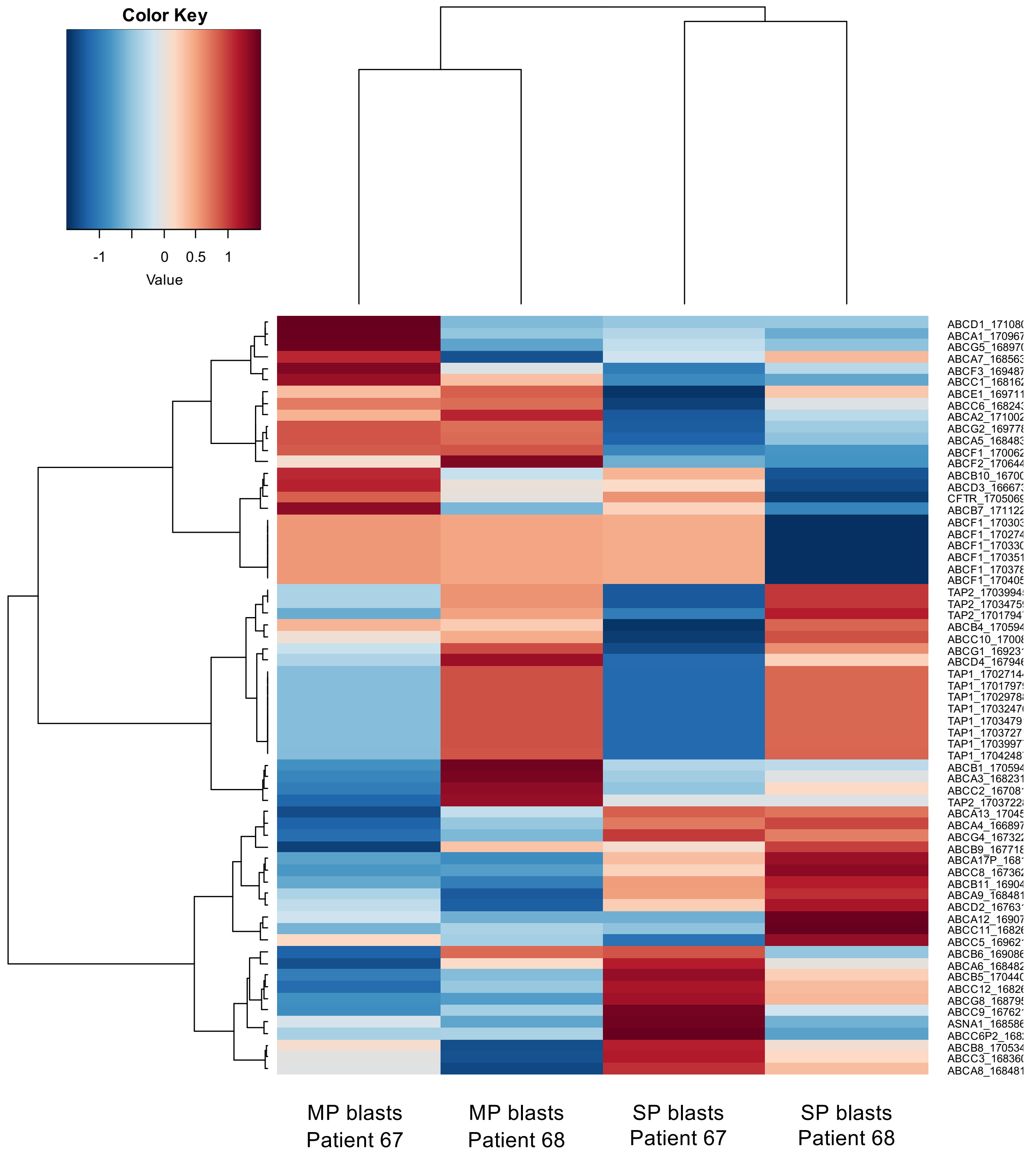

Figure S5: Transcriptomic analysis of ABC transporters in SP and MP from AML patients

Heatmap represents the expression level of ABC transporters obtained by transcriptomic analysis.

Figure S6

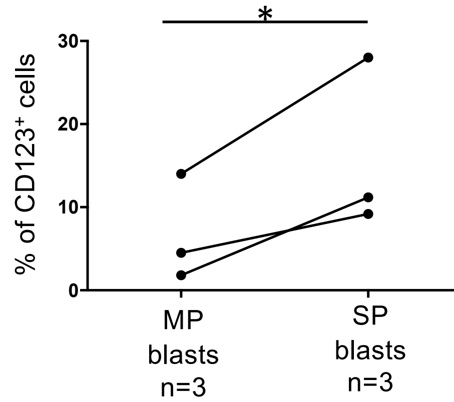

Figure S6: Quantification of $CD123^+$ cells in SP and MP blast population

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).