SUPPLEMENTARY APPENDIX

A three-dimensional *ex vivo* tri-culture model mimics cell-cell interactions between acute myeloid leukemia and the vascular niche

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A three-dimensional *ex vivo* tri-culture model mimics cell-cell interactions between acute myeloid leukemia and the vascular niche

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Supplementary Materials and Methods

3D leukemia constructs

MMP-degradable starPEG (PEG-MMP) peptide and heparin-(maleimide)$_4$ conjugates were prepared as described previously.[1, 2] Mono-culture experiments utilized gels at a crosslinking degree of γ0.75, γ1.0 or γ1.5, which equate to a stiffness of approximately 500, 1500 and 3000 Pa, while tri-culture experiments utilized gels at a crosslinking degree of γ0.63 (200-300 Pa).[2] Stiffer hydrogels were applied for mono-culture experiments as the higher density of leukemia cells promoted faster degradation of the hydrogel in comparison to the tri-cultures, where softer hydrogels were required for capillary formation.[2] KG1a, MOLM13, MV4-11 or OCI-AML3 cells at a density of 1×10^6/mL hydrogel were seeded separately into non-functionalized starPEG-heparin hydrogels. For casting, 0.5 mg heparin-maleimide and 0.3 mg starPEG were each dissolved in 11 µl phosphate buffered saline (PBS). Afterwards, the components were mixed by pipette, and a 20 µl gel was cast onto a microscope slide coated with Sigmacote® (Sigma Aldrich, Steinheim, Germany). The hydrogels were calculated to have a height of 2.5 mm, width of 6 mm and volume of 20 mm^3. Primary donor AML cells (thawed from frozen vials) were seeded at a density of 2×10^6/mL hydrogel.

For AML tri-cultures, the heparin-maleimide fraction of the hydrogel was first functionalized with 2 moles of RGD-SP (H_2N-GCWGGRGDSP-$CONH_2$; molecular weight 990 g/mol; synthesized within our laboratory) per mole of heparin and vortexed thoroughly. The ratios of growth factors and cell populations were chosen based upon previous optimization performed within our laboratory[3] where we discovered that the 10% ratio of MSCs to HUVECs was optimal for formation and stabilisation of the HUVEC network in co-culture. We further tested this ratio in tri-culture with breast and prostate cancer cell lines and found it suitable for this purpose.[2] 5 µg/mL of each VEGF (Peprotech, Hamburg, Germany), FGF-2 and SDF-

1 (both Miltenyi Biotec, Bergisch Gladbach, Germany) were then added to the heparin-maleimide solution. AML cell lines were afterwards seeded at a density of 1×10^6/mL of gel, while pAML samples were seeded at a density of 2×10^6/mL of gel, in combination with 6×10^6 HUVECs and 6×10^5 MSCs per mL of gel for tumor angiogenesis experiments, into the heparin fraction before mixing with the starPEG (1:1 ratio) to create a γ0.63, 200-300 Pa hydrogel. AML only constructs were grown for 7 days as floating droplets in medium suitable for each cell type. AML tri-cultures were grown in ECGM for 14 days before fixation with 4 % paraformaldehyde. Matrigel™ (BD Biosciences, Heidelberg, Germany) or tissue culture plastic (TCP) was used as a control for all mono-culture hydrogel experiments. Light microscopy images were analyzed using Image J software (NIH).

Chemotherapeutic and signaling inhibitor experiments

AML 2D mono-cultures, 3D mono-cultures and 3D tri-cultures were tested for chemotherapeutic response to AraC and DNR. A dose response curve was first calculated. Cytosine β-D-arabinofuranoside (AraC, Cytarabine, dissolved in double distilled water, ddH_20; Sigma Aldrich) was added to cultures at 0, 5, 20, 100, 500, 1000 and 2000 μM for 24 hours before a media change. Daunorubicin hydrochloride (DNR, dissolved in ddH_20; Sigma Aldrich) was added to cultures at 0, 0.01, 0.1, 0.2, 1, 2.5, 5 μM for 24 hours before a media change. For both drugs, cell viability was determined at day 5 post-treatment using a PrestoBlue assay (described in detail in Supplementary Materials and Methods). For signaling inhibitor experiments, AMD3100 (dissolved in ddH_20; Sigma Aldrich) was applied to cultures at 0 or 2.5 μg per mL in ECGM. Sorafenib (dissolved in dimethyl sulfoxide; Santa Cruz, Heidelberg, Germany) was added to cultures at 0 or 1 μM. Cultures were treated with AMD3100 or Sorafenib for 7 days, medium was exchanged every second day for fresh medium with fresh drug solution.

Drug combination experiments

For drug combination experiments, 2.5 μg/mL AMD3100 or 1 μM Sorafenib was applied to the cultures for 48 hours, after which the medium was changed and 0.2 μM DNR was added. After 24 hours, medium was again changed and cultures were analysed 5 days post-treatment using PrestoBlue. To mimic clinical AML induction therapy in patients,[4] AraC was applied to the tri-cultures continuously for 7 days at 30 μM (7.1 μg/mL). On days 3-5, DNR was also applied to the tri-cultures at a concentration of 5.6 μM (3.2 μg/mL) for 2 hours per day. As a control, DNR was applied at the same concentration for the same duration without AraC treatment. To mimic AML induction therapy in murine models,[5, 6] AraC was applied to the tri-cultures continuously for 5 days at 424 μM (103.5 μg/mL). In addition, on days 1-3 of the AraC treatment, DNR was applied to cultures continuously at a concentration of 5 μM (3.1 μg/mL). The constructs were analysed by PrestoBlue five days and 14 days post-treatment to check for any recovered cells.

PrestoBlue Assay

As described previously,[2] a 1:10 dilution of PrestoBlue Reagent (Life Technologies) in medium was applied to each sample in a volume of 300 μl per well in a 24-well plate. Samples were incubated for 45 min at 37 °C before 100 μl of solution was aliquoted in duplicate into a black 96-well plate. Fluorescence was detected using a Tecan GENios plate reader (Tecan Deutschland GmbH, Mainz-Kastel, Germany) at a wavelength of 590 nm.

Immunostaining

After fixation with 4% paraformaldehyde in PBS, cultures were washed in PBS and then directly immunostained as a construct or embedded in OCT solution overnight in the fridge.

Samples were then snap-frozen on dry ice. Non-specific binding was blocked using PBS containing 5% goat serum (Life Technologies) and 0.1% Triton X-100 (Sigma Aldrich). Expression of CD31 (BD Biosciences), CD44, CD45, CD54, and Integrin α4 (all Biolegend, Fell, Germany) was detected using a dilution of 1:50 - 1:100 of primary antibody incubated overnight at 4 ˚C. After washing three times, a secondary Alexa Fluor 488 goat anti-mouse or anti-rabbit antibody (Life Technologies) was applied to the samples at a 1:200 dilution in combination with Phalloidin Alexa Fluor 633 (1:50 dilution; Life Technologies) overnight at 4˚C. Afterwards, samples were washed three more times and incubated with Hoechst 33342 nuclear dye (Life Technologies) at a concentration of 1 ng/mL for 30 minutes before visualisation on a Leica Confocal SP5 microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Image analysis was performed using ImageJ software (NIH). 'Vascular contact' was defined as the length (in μm) of vascular network (HUVECs and MSCs) that were in contact with CD45 positive AML cells. For quantification of the HUVEC-MSC network density, 200 μm deep confocal z-stacks stained with f-actin and CD45 were opened in ImageJ, binerized and the 3D geometrical volume calculated using the 3D analysis plugin. For final calculations, the volume of CD45 positive images was subtracted from the f-actin volume to eliminate the leukemia cells. Alternatively, binerized z-stacks were converted to maximum projection images in ImageJ and then analysed using AngioTool (leukemia cells were not excluded).[7]

Flow cytometry

2D AML cultures were harvested and centrifuged directly for flow cytometry analysis. Hydrogels containing 3D AML cultures were harvested using 2 mg/mL collagenase NB4G (SERVA, Heidelberg, Germany) for 30 minutes at 37 °C to dissolve the hydrogel. The cells were washed with MACS Running Buffer (Miltenyi Biotec), centrifuged and resuspended in

Accutase solution (Life Technologies) for 15 minutes. Cells were washed in MACS Running Buffer, centrifuged and then resuspended in MACS Running Buffer for flow cytometry analysis. The following mouse anti-human antibodies were utilized: CD29 allophycocyanin (APC), CD33 fluorescein isothiocyanate (FITC), CD34 FITC, CD45 FITC, CD49d phycoerythrin (PE), CD49e PE, CD54 PE, CD62P PE, CD106 FITC, CD117 APC, CD184 APC and HLA-DR PE (all Miltenyi Biotec). Antibodies were added at manufacturer's recommended concentrations. Forty thousand events were collected on a MACSQuant Flow Cytometer (Miltenyi Biotec) and all resulting data was analysed using FlowJo (Tree Star Inc., Ashland, USA).

Methylcellulose and migration assay

Both 2D and 3D AML cultures were harvested as described under *Flow Cytometry*. Cells were added to Methocult (Stem Cell Technologies) to achieve a final concentration of 4×10^3 /mL and plated as 1 mL per well in a 6 well plate in triplicate. Wells not used for Methocult were filled with PBS to prevent drying of the Methylcellulose. Cultures were maintained for 10 days before colonies were counted using light microscopy.

For migration studies, cells were added at 150,000 cells/100 µl in serum-free medium to a Transwell insert (Corning, purchased from Sigma Aldrich) with a 6.0 µm pore size. 100 nM SDF-1 was added to the lower compartment in 600 µl serum-free medium. Culture was maintained for 24 hours before the cells within the lower compartment were counted via MACS Quant. Results were expressed as percentage of initial seeding density.

Drug binding and release experiments

All organic solvents were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). Ultra-high purity (UHP) water was prepared to 18.2 MΩ cm at 25 ˚C on a Milli-Q® Integral System (Merck Millipore, Darmstadt, Germany). PBS was prepared by dissolving PBS tablets (Sigma-Aldrich) in UHP water which was supplemented with 0.02% (20 mg per 100 mL) sodium azide (NaN_3, Sigma-Aldrich) to prevent bacterial growth. PBS was used at 1x concentration unless otherwise specified. RPMI with 1% PS was used as solvent for the binding and release studies of the drugs DNR, AraC and Sorafenib. For AMD3100, PBS supplemented with 0.002% NaN_3 was used due to the lack of a specific absorption wavelength and signal interference with medium compounds during analysis. Collagenase NB 4G (SERVA) for gel degradation was prepared in PBS with Ca^{2+} and Mg^{2+} (Thermo Fisher Scientific, Schwerte, Germany) at 2 g/l (DNR, AraC and Sorafenib) or 0.25 g/l (AMD3100), respectively. All reagents were used without preliminary purification.

MMP-cleavable PEG-heparin hydrogel droplets were produced onto polished Sigmacote® treated glass coverslips at a crosslinking degree of γ1 with a solid content of ca. 4.5% (45 g/l) as described elsewhere;[1] final volume was 20 µl per gel according to the gel volume used for cell culture. Drug concentrations for uptake experiments were: AraC 30 µg/mL, DNR 5.7 µg/mL, Sorafenib 0.5 µg/mL and AMD3100 200 µg/mL. After gel formation in a humidified chamber, hydrogels were transferred to 2 mL microcentrifuge tubes filled with 1 mL drug uptake solution by help of a small spatula and incubated for 24 h (AraC and DNR) or 48 h (AMD3100 and Sorafenib) at 37 °C in a ThermoMixer® C (Eppendorf, Hamburg, Germany). Vials were periodically mixed at 1200 rpm for 5-10 s to remove condensed water from the inner walls back to solution. As control samples, medium (or PBS for AMD3100) with drug as well as gels incubated in medium (or PBS for AMD3100) without drug supplementation were treated in the same manner. Next, hydrogels (incubated with or without drug) were

carefully transferred to 2 mL microcentrifuge tubes filled with 1 mL collagenase solution by help of two small spatulas. To avoid residual incubation liquid being transferred to the collagenase solution, one spatula was used to grab the gels from the vial's bottom and the other one for sliding the gel carefully into the collagenase solution. Residual incubation solution was washed off the spatula afterwards into the initial vial with UHP water. Gel degradation was performed for 24 h at 37 °C in a ThermoMixer® C. Vials were periodically mixed at 1200 rpm for 5 s to remove condensed water from the inner walls back to solution. As control sample, plain collagenase (in PBS with Ca^{2+} and Mg^{2+}) was incubated in parallel. All sample solutions were frozen at -80 °C (dry ice) by help of an aluminum rack, closed with a separate microcentrifuge cap with two small holes (Ø ca. 1 mm, prepared by punching with a cannula). After freeze-drying for at least 48 h, sample tubes were closed well and stored at -20 °C upon analysis.

Analytical high performance liquid chromatography (HPLC) – DNR and Sorafenib

HPLC experiments were performed on an Agilent 1100 Series unit (Agilent Technologies, Santa Clara, USA) equipped with degasser, binary pump, autosampler, and UV/VIS diode array detector (1 cm path length cell). Mobile phases were: 5% acetonitrile/95% UHP water with 0.1% formic acid (solvent A) and 95% acetonitrile/5% UHP water with 0.1% formic acid (solvent B), respectively. Freeze-dried samples were dissolved in 100 µl 20% acetonitrile / 80% water + 1% formic acid (equivalent to 10x concentrated solutions compared to the uptake/release mixtures). *DNR*: Samples were injected at 50 µl and separated by analytical Kinetex® Evo core shell C-18 column (150 x 3 mm in size, 5 µm particle size, 100 Å pore size; Phenomenex, Aschaffenburg, Germany) over 20 min using the following gradient with a flow rate of 0.5 mL/min: 0% B from 0-5 min, then 0-60% B from 5-17 min, finally 60-100% B from 17-19 min and 0% B after 20 min. For HPLC separations, the monitoring wavelength was set

to 478 nm and the drug peak of the HPLC elugram was integrated. Calibration was performed by injecting drug amounts of 100-800 ng dissolved in RPMI medium + 1% PS (injection volume always 50 µl) and peak integration. The amount of drug in the samples was calculated from the respective calibration curve. An overview of the drug calibration is given in the Supplementary Figure S6. Representative examples for DNR drug binding and release analysis are given in the Supplementary Figure S7A-B. *Sorafenib*: Samples were injected at 50 µl and separated by analytical ZORBAX Eclipse XDB C-8 column (150 x 4.6 mm in size, 5 µm particle size, 80 Å pore size; Agilent Technologies) over 17 min using the following gradient with a flow rate of 1 mL/min: 0% B from 0-2.5 min, then 0-95% B from 2.5-15 min, finally 95-100% B from 15-16 min and 0% B after 17 min. For HPLC separations, the monitoring wavelength was set to 260 nm and the drug peak of the HPLC elugram was integrated. Calibration was performed by injecting drug amounts of 10-500 ng dissolved in RPMI medium + 1% PS (injection volume always 50 µl) and peak integration. The amount of drug in the samples was calculated from the respective calibration curve. An overview in drug calibration is given in the Supplementary Figure S6. Representative examples for Sorafenib drug binding and release analysis are given in the Supplementary Figure S7C-D.

Analytical high performance size exclusion chromatography (HPSEC) – AMD3100 and AraC

HPSEC experiments were performed on the same instrument as for HPLC analysis. Mobile phase was 10x PBS with 0.02% NaN_3. Freeze-dried samples were dissolved in 100 µl UHP water (equivalent to 10x concentrated solutions compared to the uptake/release mixtures). Samples were injected at 50 µl and separated by analytical PolySep GFC P-3000 SEC column (300 x 7.8 mm in size; Phenomenex) over 20 min using a flow rate of 1 mL/min. *AMD3100*: For HPSEC separations, the monitoring wavelength was set to 230 nm and the drug peak of the HPSEC elugram was integrated. Background subtraction was performed for the degraded

gel samples after drug uptake with the respective spectra of degraded plain gel samples (without drug) due to an overlay of the collagenase/gel and drug signals at the detection wavelength. Calibration was performed by injecting drug amounts of 1-100 µg dissolved in PBS + 0.02% PS (injection volume always 50 µl) and peak integration. The amount of drug in the samples was calculated from the respective calibration curve. An overview in drug calibration is given in the Supplementary Figure S6. Representative examples for AMD3100 drug binding and release analysis are given in the Supplementary Figure S7E-F. *AraC*: For HPSEC separations, the monitoring wavelength was set to 270 nm and the drug peak of the HPSEC elugram was integrated. Background subtraction was performed for the degraded gel samples after drug uptake with the respective spectra of degraded plain gel samples (without drug) due to an overlay of the collagenase/gel and drug signals at the detection wavelength. Calibration was performed by injecting drug amounts of 1-25 µg dissolved in RPMI medium + 1% PS (injection volume always 50 µl) and peak integration. The amount of drug in the samples was calculated from the respective calibration curve. An overview in drug calibration is given in the Supplementary Figure S6. Representative examples for AraC drug binding and release analysis are given in the Supplementary Figure S7G-H.

Calculation of the observed binding constant (K_b)

The binding constant (K_b) for each drug was calculated from the drug amounts taken up by the gels measured by HPLC/HPSEC analysis. Knowing the initial drug concentration, sample volume (1 mL) and gel volume (20 µl), drug concentrations in the gel and supernatant were determined, respectively. K_b was then calculated by the following equation.[8]

$$K_b = \frac{c_g}{c_h \cdot c_s}$$

where c_g is concentration of bound drug in the gel, c_h is concentration of the hydrogel (solid = 1) and c_s is concentration of unbound drug in the supernatant solution. The drug amounts found

in the drug solutions with or without gels were in accordance to the theoretical amounts ± 5-10%, demonstrating that the substances in the drug uptake and release mixtures can be fully recovered throughout the sample processing.

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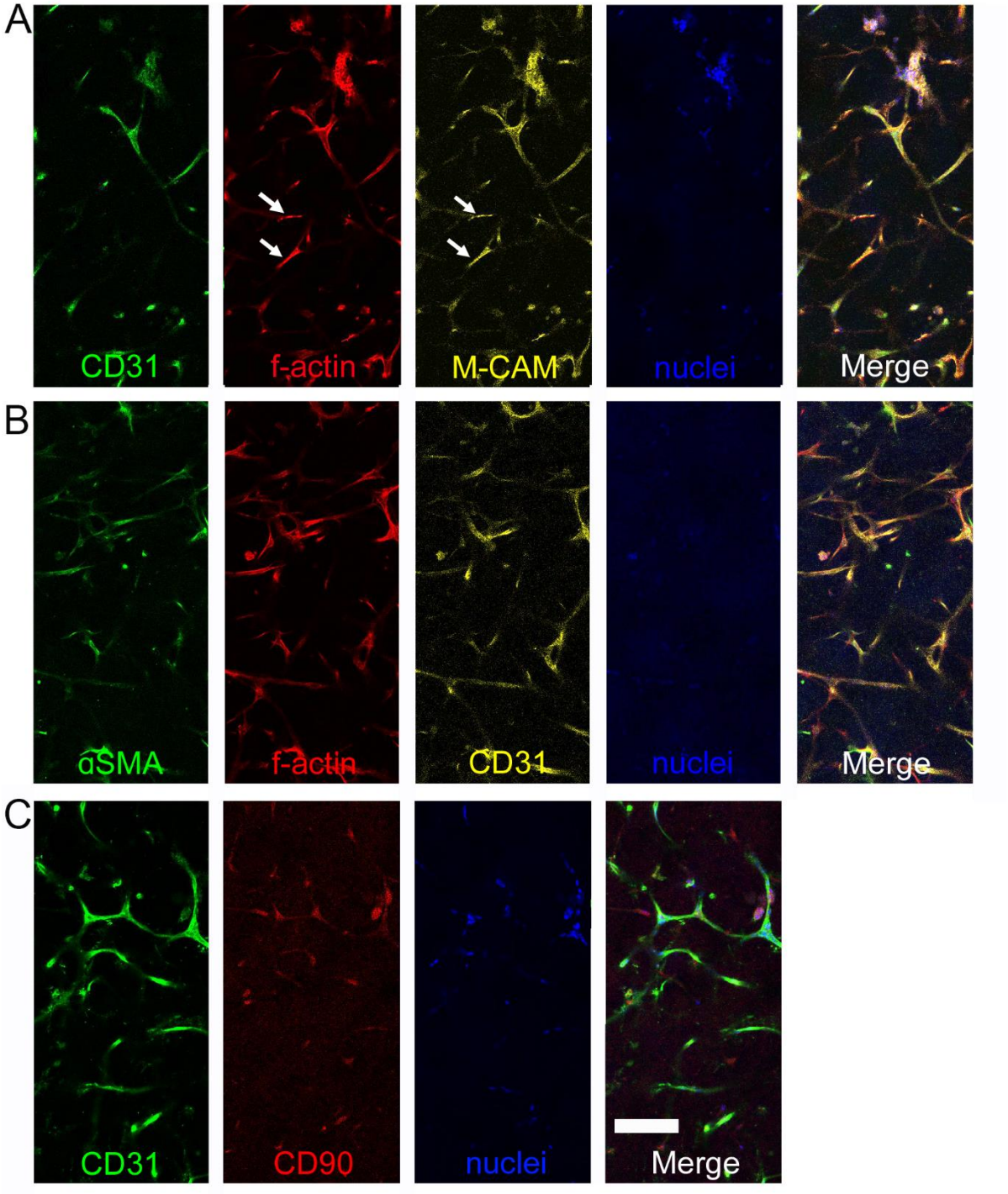
Supplementary Tables and Figures

Supplementary Table S1. Positive and negative expression of antigens on AML cell lines cultured in either 2D or 3D, as determined by flow cytometry.

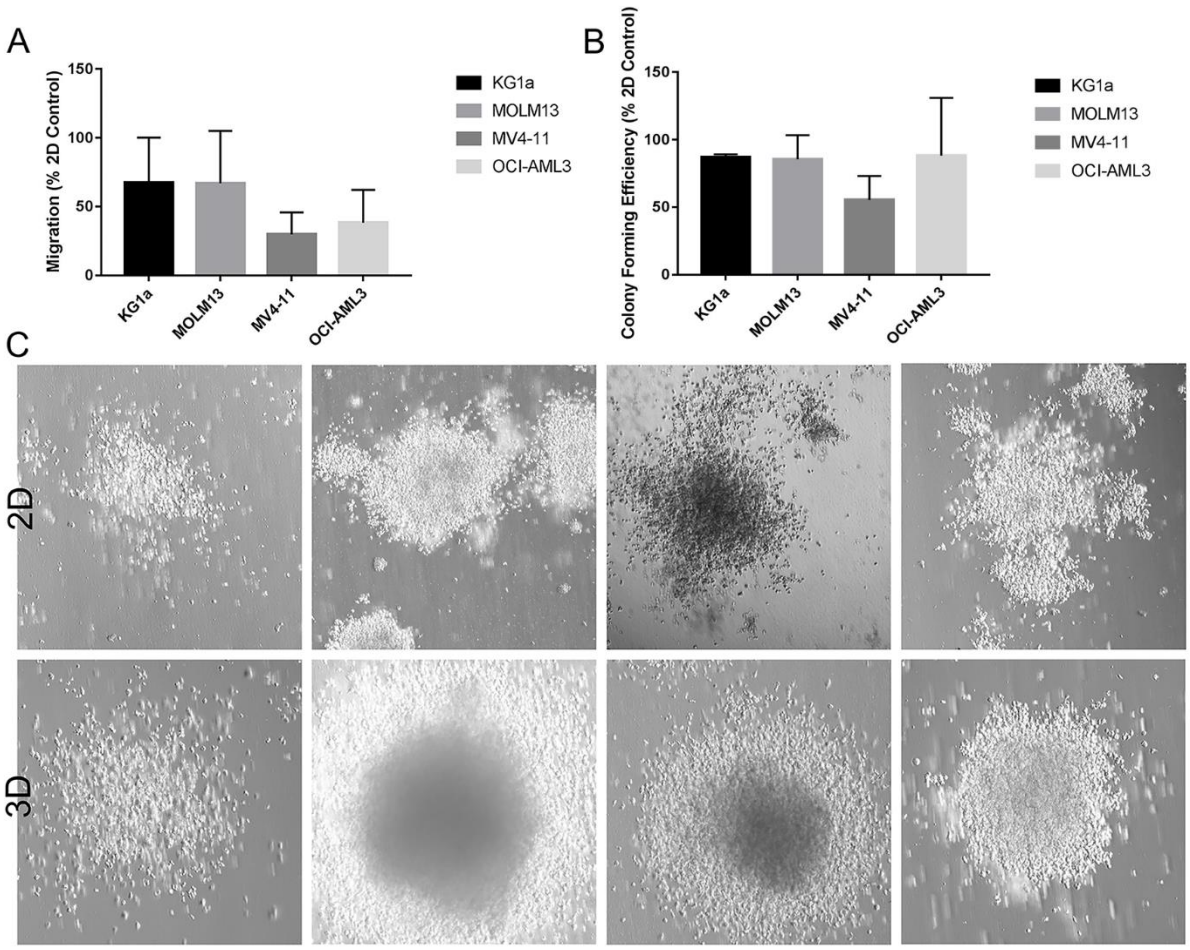
Antigen	KG1a	MOLM13	MV4-11	OCI-AML3
CD29	+	+	+	+
CD33	+	+	+	+
CD34	+	-	-	-
CD45	+	+	+	+
CD49d	+	+	+	+
CD49e	+	+	+	+
CD54	+	+	+	+
CD62P	-	-	-	-
CD106	-	-	-	-
CD117	-	-	-	-
CD184	-	-	-	-
HLA-DR	-	-	+	-

Supplementary Table S2. Patient-derived AML characteristics.

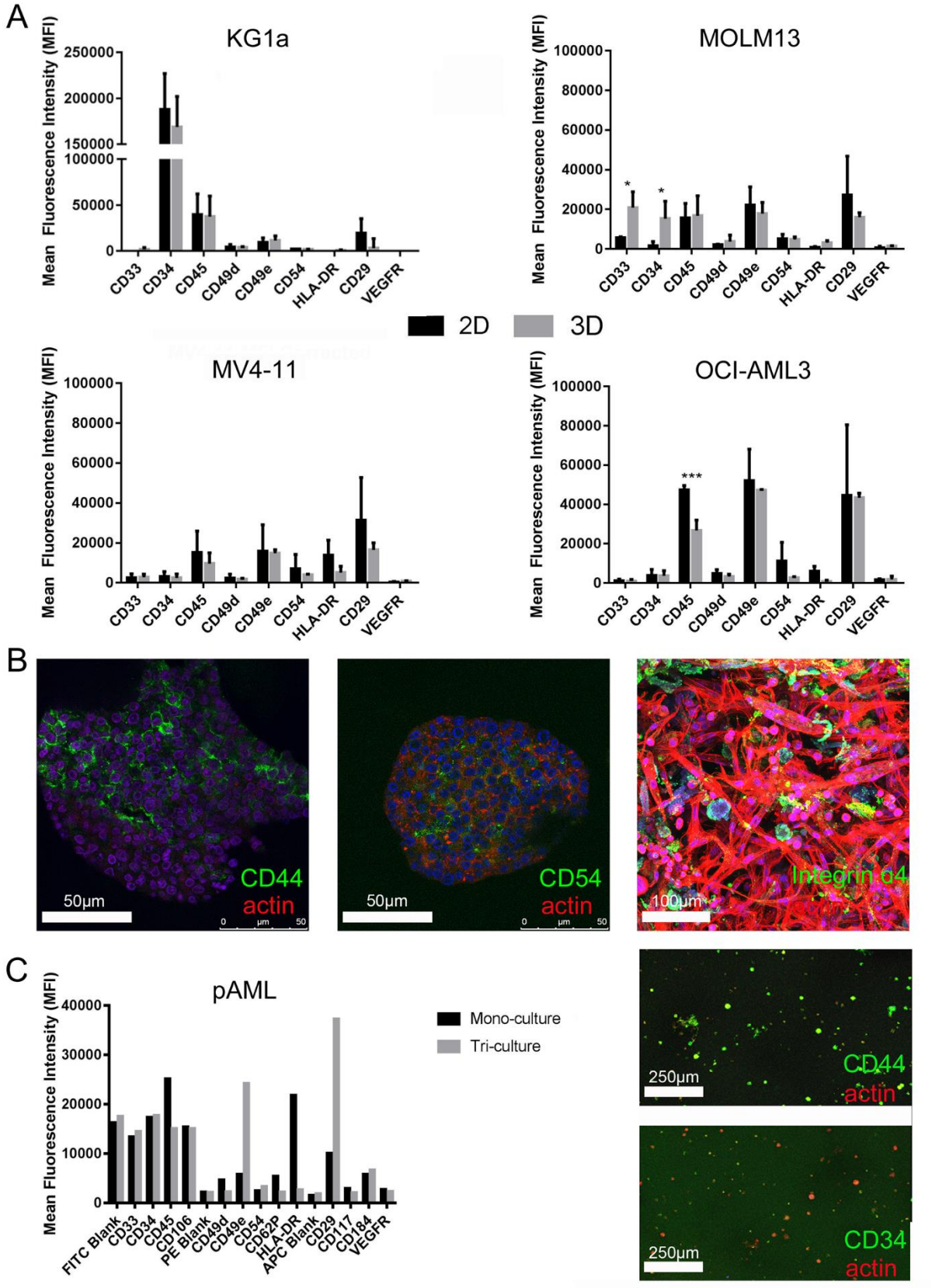
		pAML Donor 1	**pAML Donor 2**	**pAML Donor 3**
Blast	[%]	36	60	83
Source	[bone marrow vs. [peripheral blood]	bone marrow	peripheral blood	peripheral blood
Gender	[female vs. male]	male	male	male
Age	[years]	76	65	75
Status	[initial diagnosis vs. refractory vs. relapse]	initial diagnosis	initial diagnosis	refractory
FAB	classification	M2	M1	M1
WHO 2008	classification	AML with multilineage dysplasia	AML without maturation	AML with multilineage dysplasia
Cytogenetics		47,XY,+8	46,XY,del(18)	46,XY
Molecular Genetics	[only NPM1, FLT3, CEBP]	*not available*	NPM^{wt}, $FLT3^{wt}$, $CEBP^{wt}$	*not available*
ELN risk category at initial diagnosis	[fav, int-I, int-II, adv]	intermediate-II	intermediate-II	*not available*



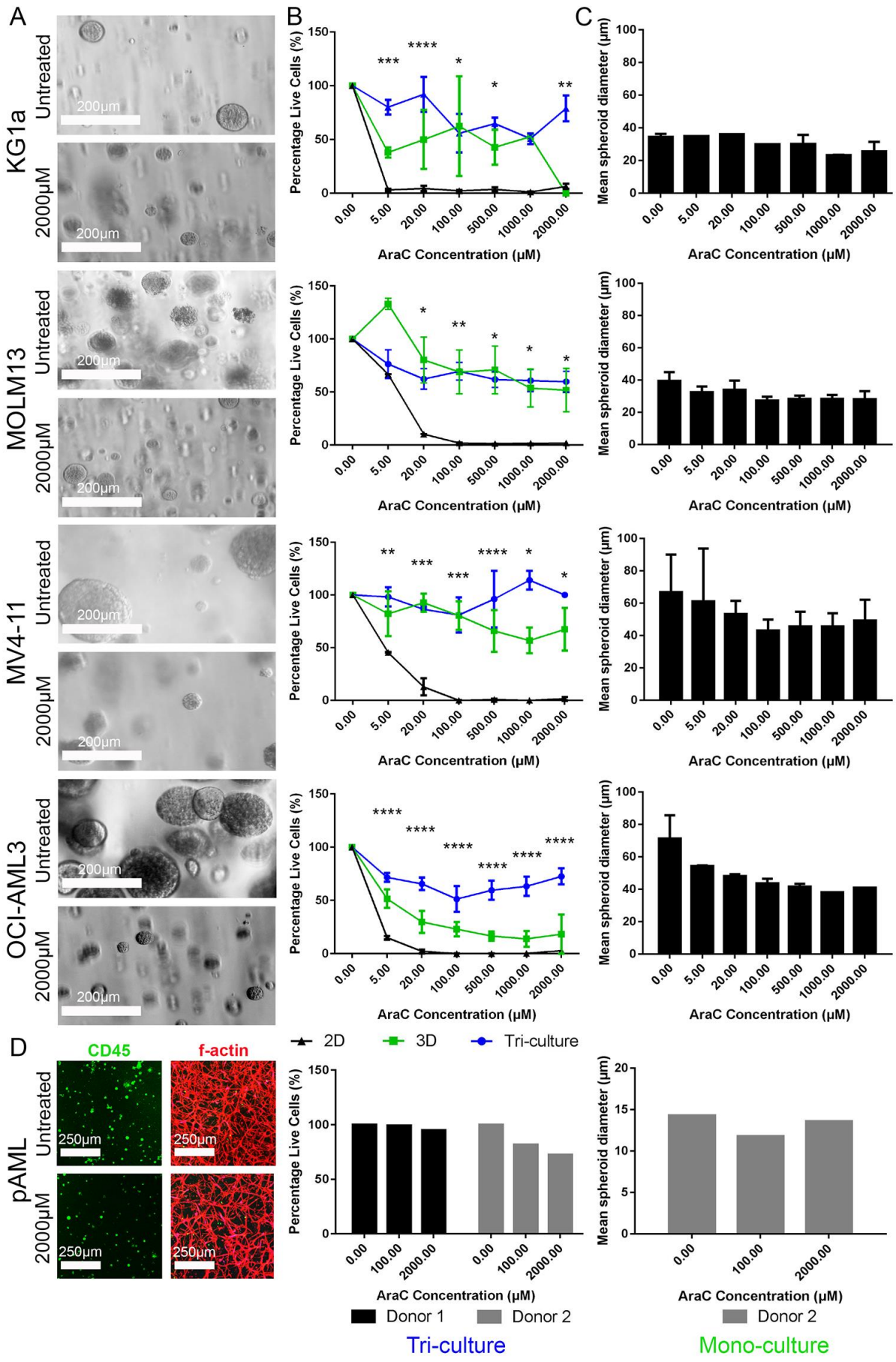
Supplementary Figure S1. Immunofluorescent images of the HUVEC-MSC vascular network in AML tri-cultures. Confocal images of HUVEC and MSC in AML tri-cultures after 7 days of culture. A) Immunostaining depicts CD31 (green), f-actin (red), M-CAM (yellow), and nuclear staining (DAPI, blue). Arrows indicate M-CAM^{+}CD31^{-} cells. B) Immunostaining depicts αSMA (green), f-actin (red), CD31 (yellow), and nuclear staining (DAPI, blue). C) Immunostaining depicts CD31 (green), CD90 (red), and nuclear staining (DAPI, blue). Scale bar = 100μm.



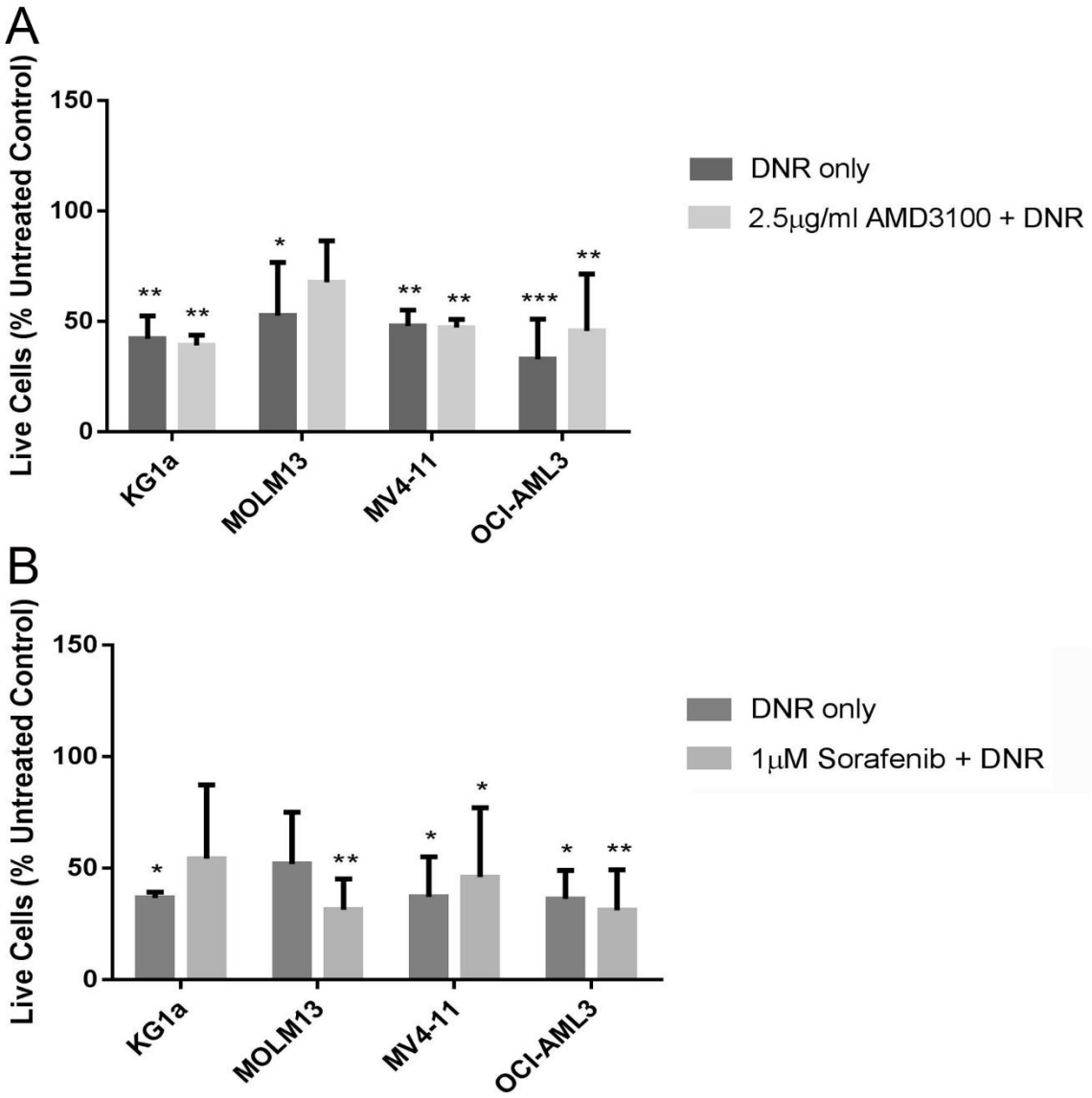
Supplementary Figure S2. Functionality of AML cells cultured in 3D. A) Percentage of 3D cultured Transwell migrating cells in response to SDF-1, when compared with 2D cultured cells after 24 h. In some cases, reduced migration was demonstrated in 3D cultured cells than in 2D cultured cells. This may be due to residual hydrogel pieces blocking the Transwell pores after enzyme treatment to remove the cells from the hydrogel. B) Percentage of 3D pre-cultured colonies formed in Methylcellulose when compared with 2D pre-cultured colonies. Experiments were performed twice in duplicate (n=2). Graphs display mean ± SD. C) Light microscope images of 2D (upper row) and 3D (lower row) cultured AML cells cultured within Methylcellulose after 14 days. No significant differences in colony formation were visualized between cells pre-cultured in 2D versus those pre-cultured in 3D.


A
KG1a
MOLM13
MV4-11
OCI-AML3
Mean Fluorescence Intensity (MFI)
CD33
CD34
CD45
CD49d
CD49e
CD54
HLA-DR
CD29
VEGFR
2D
3D
B
CD44
actin
50μm
CD54
actin
50μm
Integrin α4
100μm
C
pAML
Mono-culture
Tri-culture
FITC Blank
CD33
CD34
CD45
CD106
PE Blank
CD49d
CD49e
CD54
CD62P
HLA-DR
APC Blank
CD29
CD117
CD184
VEGFR
CD44
actin
250μm
CD34
actin
250μm

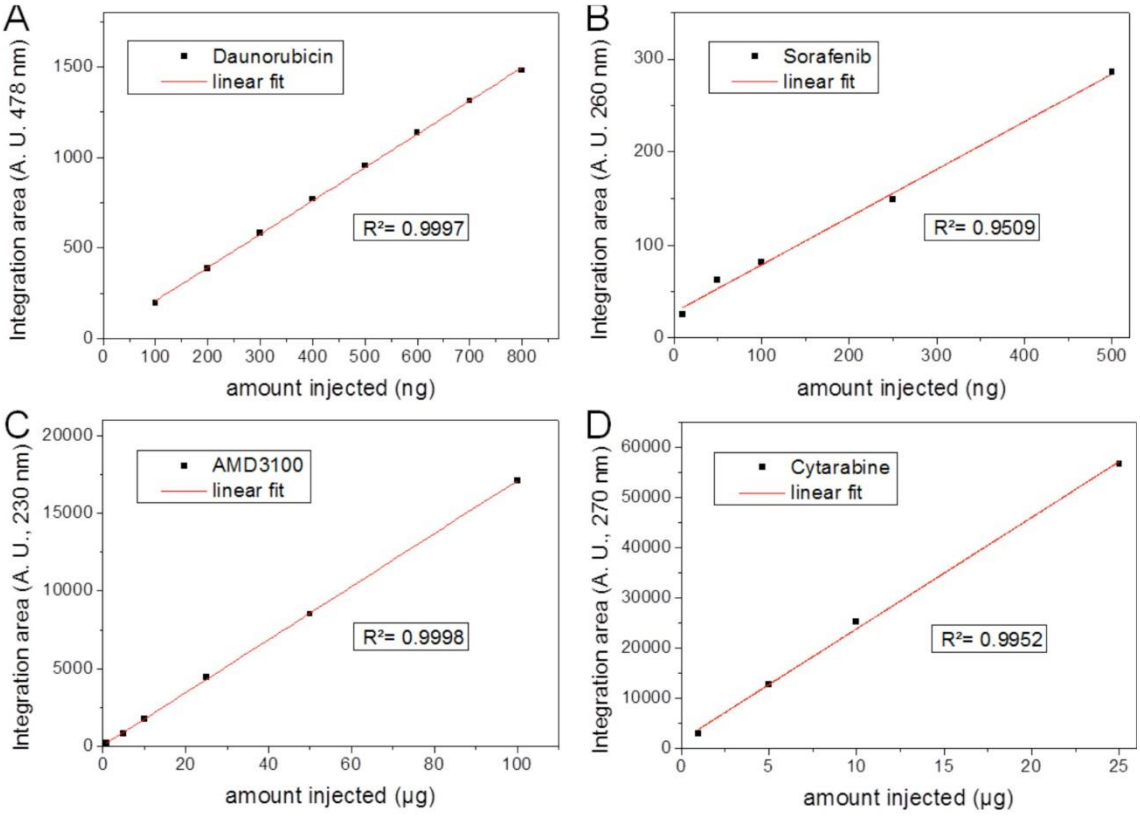
Supplementary Figure S3. Phenotype of AML cells cultured in 3D. A) Mean fluorescence intensity of phenotypic markers on 2D and 3D cultured AML cells as determined by flow cytometry. Experiments were performed twice in duplicate (n=2). Graphs display mean ± SD. Some differences in selected adhesion ligands were visualized in cells cultured in 2D when compared with those cultured in 3D. B) Representative confocal images of AML cell line monocultures showing CD44 (left) and CD54 (centre) in green as well as AML tri-cultures showing integrin alpha 4 (right) in green. C) Mean fluorescence intensity of phenotypic markers on pAML from one patient cultured in 3D monocultures and tri-cultures (left). Experiment was performed once in duplicate (n=1). Graphs display mean only. Expression of CD44 and CD34 in pAML 3D monocultures as shown by confocal microscopy. Variations in CD49e, HLA-DR, and CD29 were visible between 2D and 3D pAML cultures. *Indicates statistical significance between 2D mono-culture and 3D tri-cultures: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.


A
B
C
KG1a
MOLM13
MV4-11
OCI-AML3
Untreated
2000μM
200μm
Percentage Live Cells (%)
Mean spheroid diameter (μm)
AraC Concentration (μM)
0.00
5.00
20.00
100.00
500.00
1000.00
2000.00
2D
3D
Tri-culture
D
CD45
f-actin
pAML
250μm
Donor 1
Donor 2
Tri-culture
Mono-culture

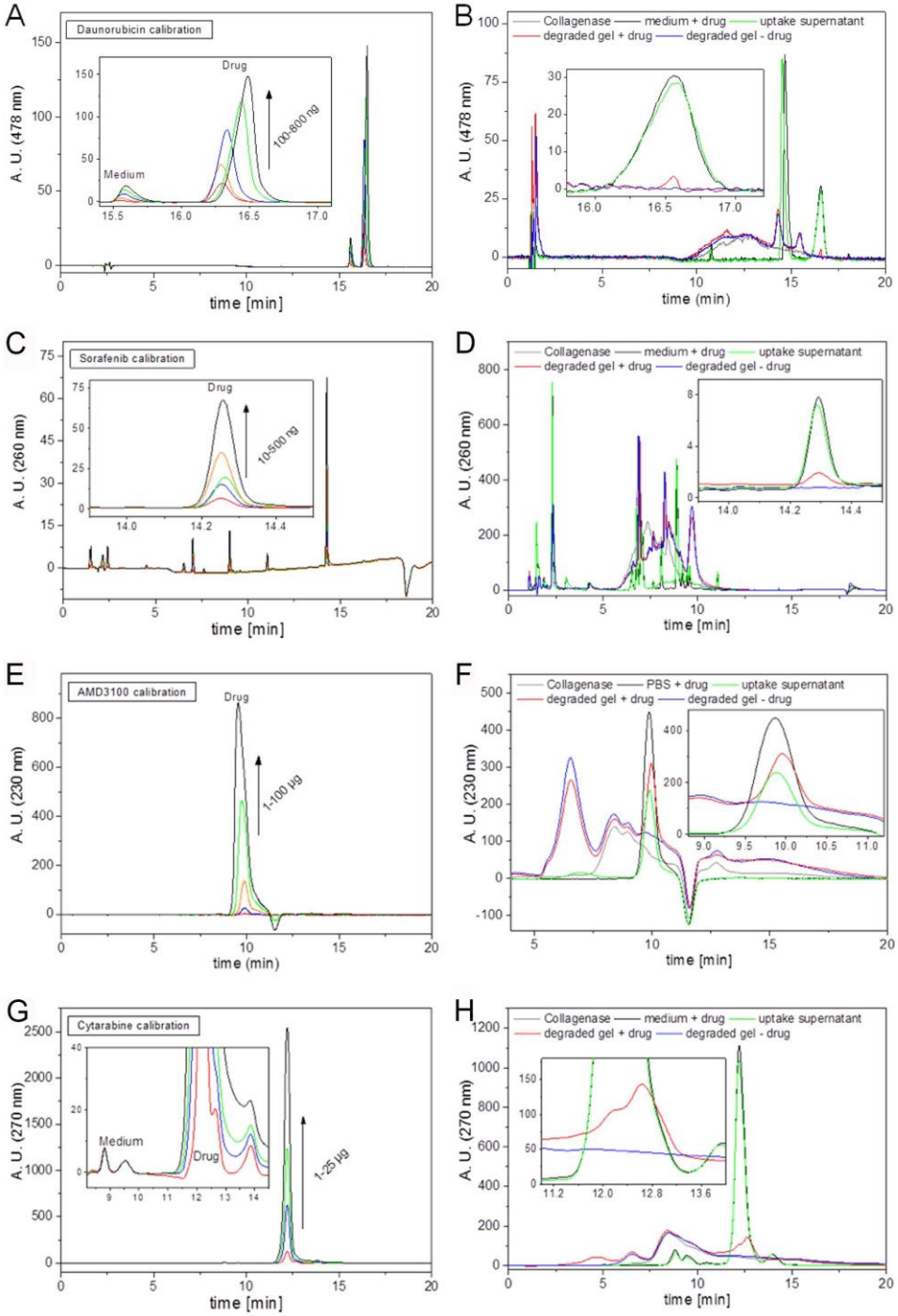
Supplementary Figure S4. Treatment of 3D mono-cultures and tri-cultures with AraC. A) Light microscope images of AML cell lines untreated and treated with 2000 μM AraC at day 7 post-treatment. B) AML cells lines were treated with six concentrations of AraC as 2D mono-cultures, 3D mono-cultures or 3D tri-cultures. The doses of AraC utilized were not effective on 3D cultures with the exception of OCI-AML3 mono-cultures. Graphs display mean ± SEM. C) Mean spheroid diameter of 3D mono-cultures after treatment with AraC (± SD). D) pAML from a patient with AML were cultivated as 3D mono-cultures (left confocal images) or 3D tri-cultures (right confocal images). Mono-cultures (right graph) and tri-cultures (left graph) were treated with two concentrations of AraC. Graph displays mean only. 3D mono-cultures of pAML did not produce enough metabolic activity to show effects of AraC. Therefore the percentage of live cells is displayed from tri-cultures only. Cell line experiments were performed at least 3 times in triplicate (n=3-4). pAML experiments were performed one time in triplicate. *Indicates statistical significance between 2D mono-culture and 3D tri-cultures: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.



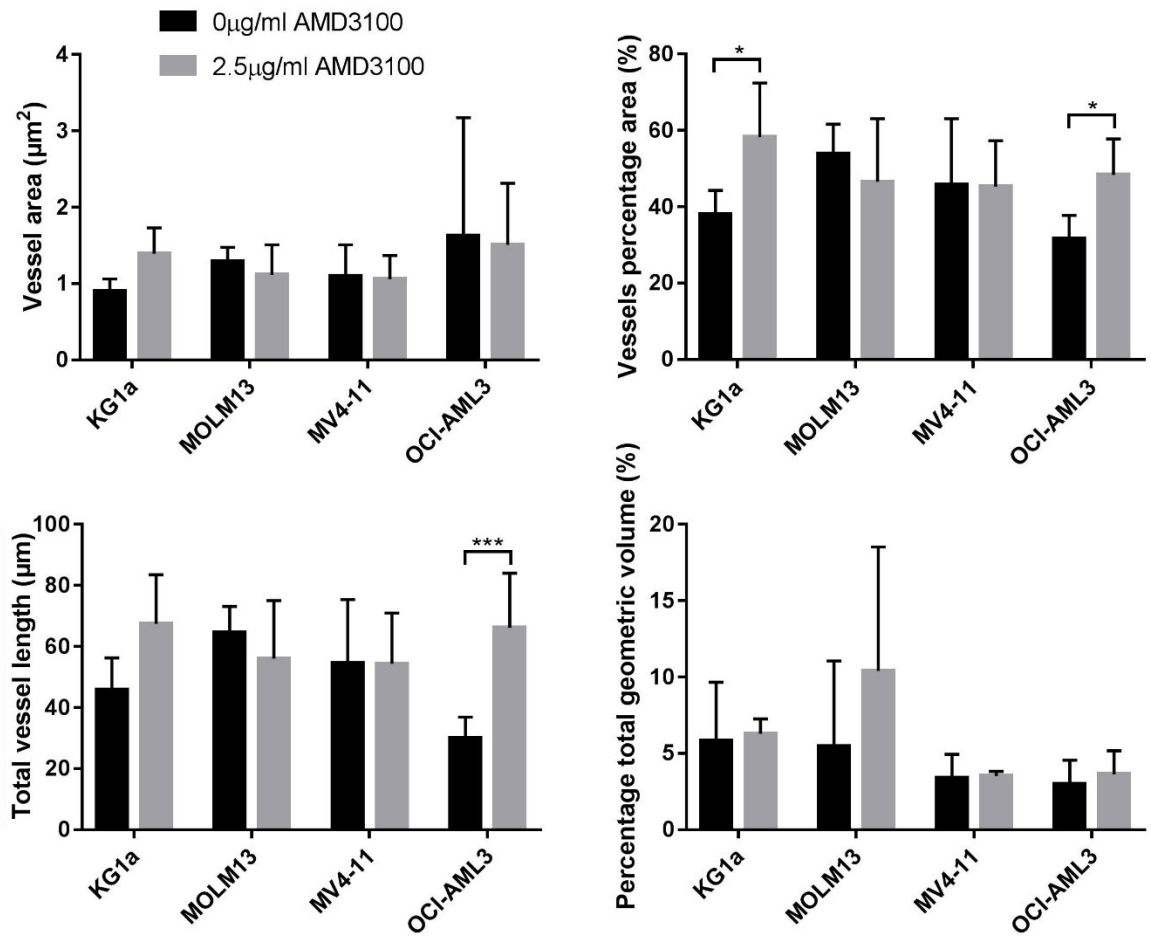
Supplementary Figure S5. Combination treatment of AML tri-cultures. A) Percentage of live cells after treatment with DNR only or with 2.5 μg/mL AMD3100 pre-treatment. B) Percentage of live cells after treatment with DNR only or with 1 μM Sorafenib pre-treatment. Experiments were performed twice in duplicate (n=2). Graphs display mean ± SD. It was demonstrated that pre-treatment of the 3D tri-cultures with AMD3100 or Sorafenib did not increase DNR efficacy. *Indicates statistical significance between untreated control and treated cultures: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



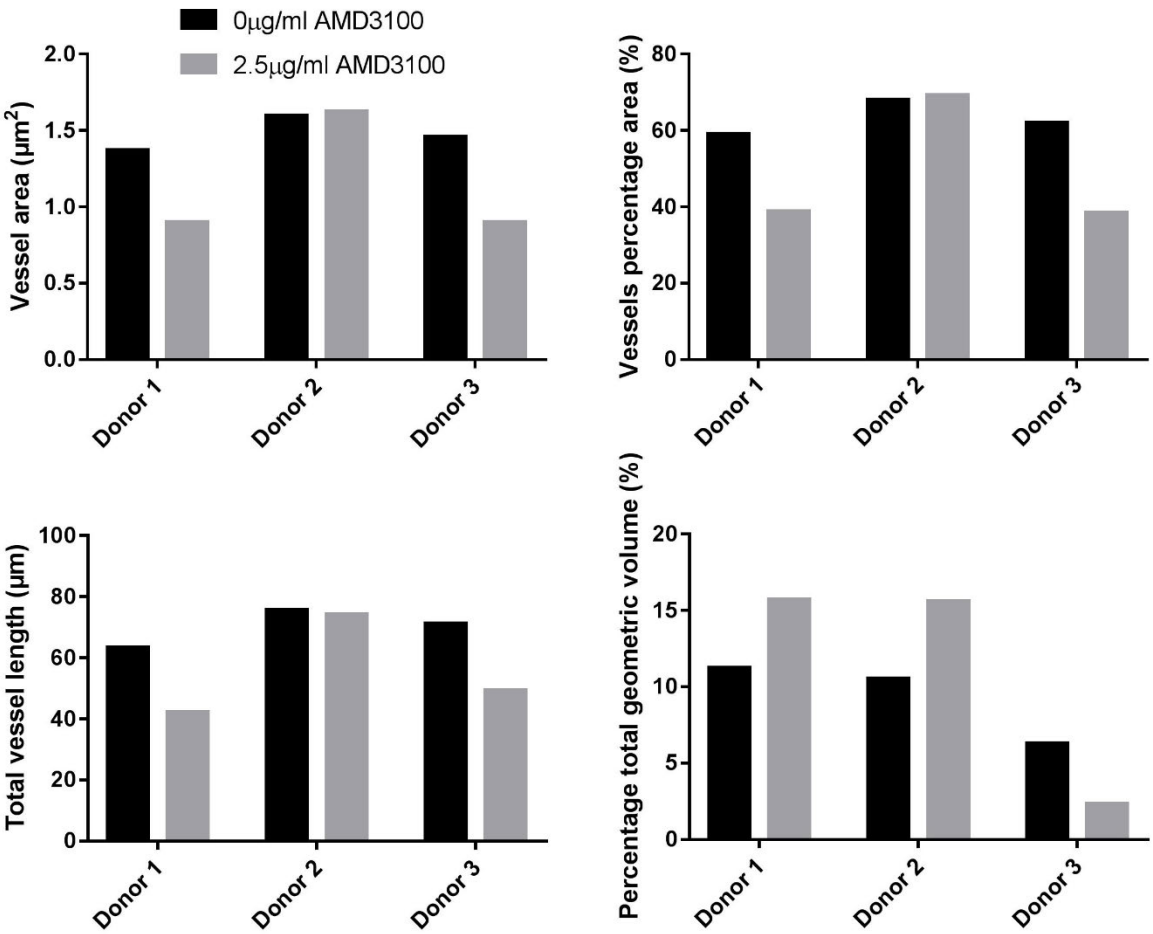
Supplementary Figure S6. **Calibration curves generated from peak integration of the respective HPLC/HPSEC elugrams for the drugs (a) Daunorubicin (DNR), (b) Sorafenib, (c) AMD3100 and (d) Cytarabine (AraC).** A.U. = arbitrary units.



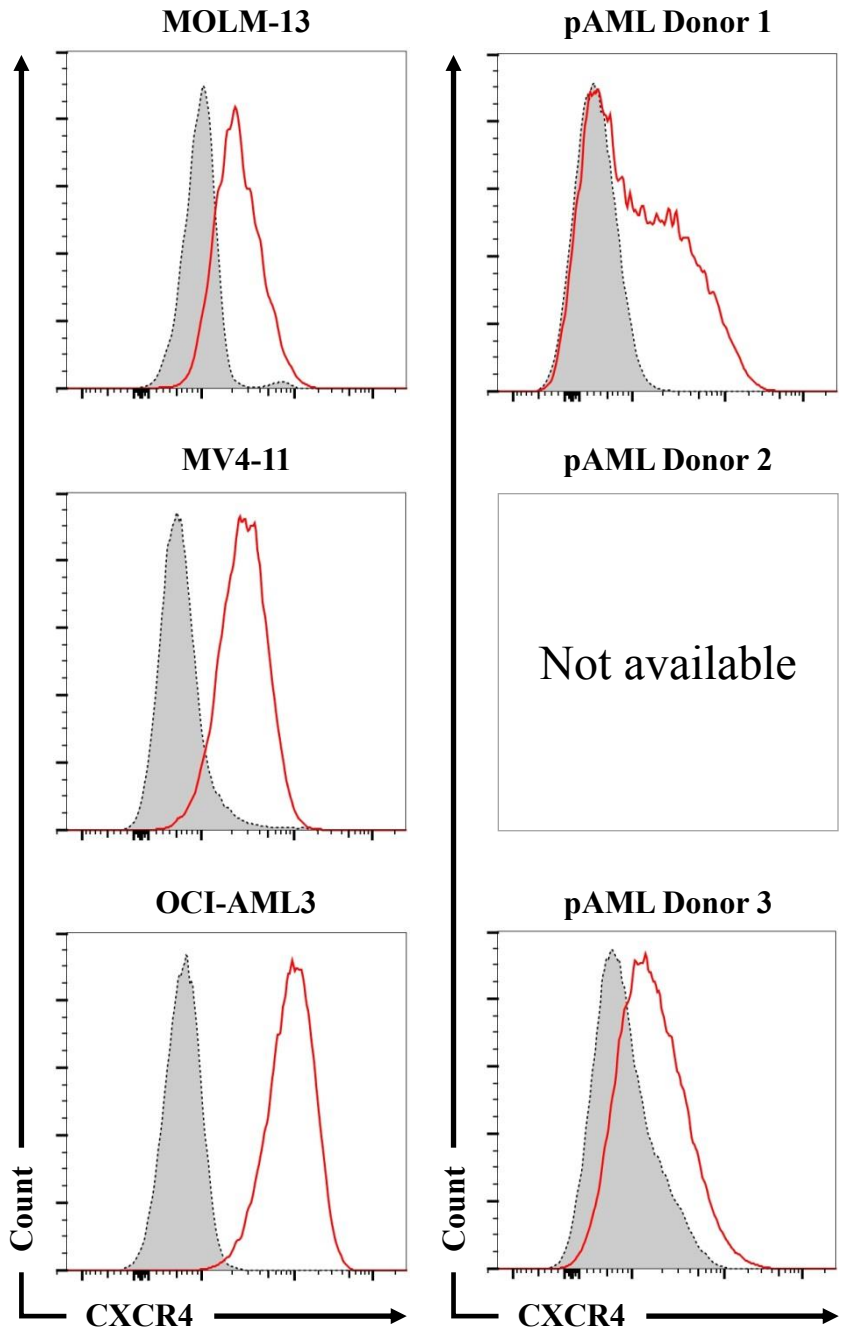
Supplementary Figure S7. **HPLC and HPSEC analysis of hydrogel drug uptake and release experiment.** HPLC elugrams of increasing DNR (a) and Sorafenib (c) drug amounts dissolved in RPMI + 1% PS. b, d) HPLC elugrams of collagenase solution (grey), drug solution before (medium + drug, black) and after (uptake supernatant, green) hydrogel incubation as well as the degraded gel incubated with (red) or without (blue) drug supplementation. (e) HPSEC elugrams of increasing AMD3100 drug amounts dissolved in PBS + 0.02 % NaN_3. (g) HPSEC elugrams of increasing Cytarabine drug amounts dissolved in RPMI + 1% PS. (f, h) HPSEC elugrams of collagenase solution (grey), drug solution before (medium + drug, black) and after (uptake supernatant, green) hydrogel incubation as well as the degraded gel incubated with (red) or without (blue) drug supplementation. All injections were 50 µl. A.U. = arbitrary units.



Supplementary Figure S8. Effects of AMD3100 treatment on vascular network density in the presence of AML cell lines. Confocal z-stacks were binarized, converted to maximum projection and then analyzed using AngioTool software to assess vessel area, vessel percentage area and total vessel length. Otherwise, binarized z-stacks were analyzed using ImageJ for 3D geometric volume. The volume of CD45 leukemia cells was subtracted from the volume of f-actin staining and the data is presented as a percentage of the total z-stack volume. Experiments were performed twice in duplicate (n=2). Graphs display mean ± SD. *Indicates statistical significance between untreated control and treated cultures: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary Figure S9. Effects of AMD3100 treatment on vascular network density in the presence of pAML. Confocal z-stacks were binarized, converted to maximum projection and then analyzed using AngioTool software to assess vessel area, vessel percentage area and total vessel length. Otherwise, binarized z-stacks were analyzed using ImageJ for 3D geometric volume. The volume of CD45 leukemia cells was subtracted from the volume of f-actin staining and the data is presented as a percentage of the total z-stack volume. Experiments were performed once in triplicate (n=1). Graphs display mean.



Supplementary Figure S10. CXCR4 expression in AML cell lines and pAML cells. Histograms depicting CXCR4 expression on MOLM-13, MV4-11, OCI-AML3 and pAML donors 1 and 3, as determined by flow cytometry. Experiments were performed once in triplicate (n=1).