Dexamethasone in hyperleukocytic acute myeloid leukemia

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SUPPLEMENTAL DATA

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Supplementary Material

STATISTICAL ANALYSES

Before doing any analyses, we assessed the power of the study: 102 deaths provided a power greater than 80% to detect a Hazard Ratio (HR) for OS of ≤ 0.56 (for dexamethasone vs. the no-dexamethasone group) with a two-sided type-1 error rate of 5% (α=0.05) for the comparison of two exponential survival distributions.[1]

Statistical analyses were performed using STATA software 11.2 (STATA Corp., College Station, TX, USA). Complete response, induction failure, day-60 deaths, fungal or bacterial infections, grade 3–4 bleeding events, and admissions into an intensive-care unit during the induction phase were compared between dexamethasone and the no-dexamethasone group using the χ^2-test (or Fisher's exact test for small data sets). Univariate analyses used cumulative incidence functions and Gray's test to assess the relapses, whereas having non-relapse mortality was treated as a competing event. For survival end points, Kaplan–Meier survival curves were drawn and differences in survival functions were tested using the log-rank test. HR and 95% confidence intervals (CI) were assessed using a standard Cox model for survival end points, and a proportional sub distribution hazard model, which is an extension of the Cox model to the situation of competing risks, for the cumulative incidence of relapse.[2] Variables initially introduced into the multivariate survival analyses were all variables (potential confounding factors) associated with relapse, disease-free survival, event-free survival, or overall survival in univariate analyses with a *P*-value < 0.20. A backward analysis was then applied until only variables significantly and independently associated with relapse, disease-free survival, event-free survival, or overall survival (*P*-value <0.05) remained. The proportional-hazard assumption was tested for each covariate of the Cox model using the "log-log" plot method curves and was always met. When the linearity hypothesis was not respected, continuous variables were transformed into ordered

data. Interactions between independent covariates and dexamethasone were tested in the final models. Allogeneic stem-cell transplantation was evaluated as a time-dependent covariate. All reported *P*-values were two-sided and the significance threshold was set at <0.05.

EXPLORATORY ANALYSES

CO-CULTURES AND LONG TERM CULTURE (LTC) (3, 4)

Co-cultures of AML cell lines with MS-5 confluent monolayer were performed in RPMI1640. Co-culture of primary samples were performed in MyeloCult H5100 (StemCell Technologies, Vancouver, BC, Canada) supplemented with recombinant human IL3, G-CSF, and TPO (MS-5+3GT) (20 ng/ml each; Peprotech, London, U.K.). Cytarabine or dexamethasone treatments for 1 week were safe for the confluent MS-5 stromal cells as evidenced by DAPI staining. Cells were cultured at 37°C in 5% CO2 - humidified incubators. To determine treatments impact on Leukemic-LTC-IC, primary co-culture of patient samples were harvested by trypsinization after one week of treatment and stained with anti-Sca-1-PE and anti-CD45-APC-Cy7 antibodies as well as with DAPI. CountBright™ absolute counting beads (Molecular Probes Invitrogen, Paisley, UK) were used to assess by flow cytometry the total number of live human cells ($Sca\text{-}1^-CD45^+DAPI^-$) per condition, following manufacturer's recommendations. Precise number of live human cells was subsequently replated in limiting dilution in 20 replicates in 96-well microplates containing confluent MS-5 monolayer. Half medium change was done twice a week without disrupting the established feeders. After 5 weeks, LTC medium was replaced by methylcellulose H4435 (StemCell Technologies). After an additional 2 weeks, each well was scored as negative if no colonies were present. The frequency of L-LTC-IC was calculated using LCalc software (StemCell Technologies) according to the Poisson statistics and method of maximum likelihood.

Xenotransplantations using NOD-Scid-IL2rγ^{null} mice and transcriptomic data mining were also used to explore the antileukemic activity of dexamethasone.

BIOINFORMATICS ANALYSES

Two freely-available gene-expression data sets for AML and a gene expression profile for cytarabine-resistant leukemic cells from NOD/LtSz-scid/IL-2Rγchain null (NSG) mice (see below) were used in this study.(5-7) Lists of differentially expressed mRNA (>-/+1.5 fold change and a false discovery rate of <0.05) were uploaded into the Genome Analyzer bioinformatics tool (Genomatix, http://www.genomatix.de) for further functional analyses (gene ontology term, small molecules). This program allows characterization of large sets of genes by making use of annotation data from various sources, like Gene Ontology or Genomatix proprietary annotation. Overrepresentation of different biological terms within the input are calculated and listed in the output together with the respective *P*-values. The significance of the association between each list and functions or canonical pathways or small molecule effects was measured by Fisher's exact test. Transcriptional-gene regulatory networks were built based on the molecular relationship repertoire referenced in the Genomatix library. The Genomatix Upstream Regulator's analytic was also used to identify the transcriptional regulators that could explain the experimental gene-expression patterns, predict their activation state, and determine the biological functions affected by the regulatory cascade. Small pharmacological molecules (including dexamethasone) gene signatures were built based on the small molecule transcriptomic repertoires referenced in the Genomatix library and significantly overrepresented in our or published gene expression datasets. TCGA(5) and Verhaak(6) datasets were grouped according to genetic features (*NPM1, FLT3-ITD, FLT3-TKD, CEBPA, IDH1, IDH2*, or *RUNX1* mutations, *CBFB-MYH11, RUNX1-RUNX1T1*, or *BCR-ABL* rearrangements, complex karyotype and *MLL* fusions), when available. Each genetically-defined subgroup of AML was characterized by its enrichment in a dexamethasone gene signature.

PATIENT-DERIVED XENOGRAFT MODEL

Animals were used in accordance to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Région Midi-Pyrénées (France). NSG mice were produced at the

UMS006 (INSERM, Toulouse, France) using breeders obtained from Charles River Laboratory as reported previously.(8)

Briefly, mice were housed in sterile conditions using HEPA filtered micro-isolators and fed with irradiated food and sterile water. Transplanted mice were treated with antibiotic (Baytril) for the duration of the experiment. Mice (6–8 weeks old) were sub-lethally treated with 30 mg/kg busulfan at 24 hours before injection of AML cells from 3 patients (patient#53, normal karyotype with *FLT3*-ITD and *NPM1* mutation; patient#262, normal karyotype with FLT3-ITD mutation; patient#325, karyotype unknown with *FLT3*-D835, *NPM1* and *IDH1*-R132S mutations).(9) AML samples were thawed at room temperature, washed twice in PBS, and suspended in Hanks balanced salt solution at a final concentration of 1–5 million cells per 200 µL of solution per mouse used for tail-vein injection. Daily monitoring of mice for symptoms of disease (ruffled coat, hunched back, weakness, and reduced mobility) determined the time of killing those injected animals with signs of distress. If no signs of distress were seen, mice were initially analyzed for engraftment at 8 weeks after injection except where otherwise noted.

At 8–18 weeks after AML cell transplantation and when the mice were engrafted (tested by flow cytometry on peripheral blood), the mice received daily intraperitoneal injections of 60 mg/kg cytarabine for 5 days (kindly provided by the Pharmacy of Toulouse University Hospital France) or vehicle (phosphate-buffered saline [PBS]). Mice were monitored for toxicity symptoms and provided nutritional supplements as needed. Three days after the last dose of cytarabine or vehicle, viable human AML blasts from three patients were collected from the bone-marrow engrafted mice, purified by FACS-sorting and processed for transcriptomic analysis using microarrays covering 23,924 human genes.

CELL LINE-DERIVED XENOGRAFT MODEL

Adult mice (6–8 weeks old) were treated with 20 mg/kg busulfan (Busilvex, Pierre Fabre, France) by intraperitoneal administration at 24 hours before injection of the OCI-AML3 cell line. Cultured AML cell lines were washed twice in PBS and cleared of aggregates and debris using a 0.2-mm cell filter,

and suspended in PBS at a final concentration of 2 million cells per 200 μL of PBS per mouse for intravenous injection.

Xenograft tumors were generated by injecting OCI-AML3 cells (in 200 μL of PBS) into the tail vein of NSG mice.(10) Mice were treated on day 17 with vehicle: i.e., dexamethasone (10 mg/kg/day, 5 days), cytarabine (30 mg/kg/day, 5 days), or dexamethasone plus cytarabine (using same schedule for both drugs). Daily monitoring of mice for symptoms of disease (ruffled coat, hunched back, weakness, and reduced motility) determined the time of killing injected animals that had signs of distress. NSG mice were humanely killed in accordance with the Institutional Animal Care and User Ethical Committee of the UMS006 and Région Midi-Pyrénées Protocols. A log-rank test was used to analyze mouse survival and Kaplan–Meier survival curves were used to illustrate the results (Prism 5 software, GraphPad Software Inc., La Jolla, CA, USA).

AML SAMPLES AND CELL LINES

Samples from AML patients were obtained at Toulouse University Hospital after informed consent and were stored at the HIMIP collection (BB 0033-00060) (Supplementary Table 8). According to French law, HIMIP collections are declared to the Ministry of Higher Education and Research (DC 2008-307 collection 1) and a transfer agreement (AC 2008-129) was obtained after approbation by the “Comité de Protection des Personnes Sud-Ouest et Outremer II” (ethical committee). Clinical and biological annotations of the samples were declared to the Comité National Informatique et Libertés (i.e., Data processing and Liberties National Committee).

Peripheral blood or bone marrow samples were frozen in fetal calf serum with 10% DMSO and stored in liquid nitrogen. The percentage of blasts was determined by flow cytometry and morphological characteristics before purification. Samples with a >80% blast cell count were chosen for these studies. HL-60, MV4-11, MOLM-14, KG1a, OCI-AML2, OCI-AML3, U-937, and Kasumi cell lines were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz, Germany) and cultured in Minimum Essential Medium alpha (Thermo Fisher Scientific, Waltham, MA).

CELL VIABILITY AND APOPTOSIS ASSAY

Primary AML samples were treated for 24 hours with vehicle or dexamethasone, and then 5×10^5 cells were washed with PBS and resuspended in 300 µL of annexin-V binding buffer. Two microliters of annexin-V-FITC and 7-AAD were added for 15 min at room temperature in the dark. All samples were analyzed using a FACSCalibur flow cytometer (BD Pharmingen, San Diego, CA, USA). AML cell lines were treated for 72 hours with vehicle or 100 and 300 nM dexamethasone. A blue trypan dye exclusion assay was used to assess cell viability. Statistical analyses were performed using unpaired two-tailed Student's t-tests (Prism 5 software).

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Supplementary Tables

Table S1: Multivariate model for cumulative incidence of relapse (Fine and Gray model).

	numbers	**events**	**aSHR**	**95% CI**	***P* value**
Dexamethasone					
No	74	49	1		
Yes	50	17	0.30	0.14-0.62	0.001
AML status					
De novo	109	56	1		
Secondary	15	10	1.71	0.92-3.19	0.090
CD56 - %					
≤20	90	52	1		
>20	24	8	0.39	0.18-0.87	0.021
LDH – UI/liter					
≤1550	68	32	1		
>1550	56	34	2.21	1.30-3.77	0.003
Study period					
2004-2009	45	34	1		
2010-2015	79	39	1.93	1.04-3.58	0.036
Hydroxyurea					
No	42	28	1		
Yes	82	38	0.52	0.30-0.90	0.019
Allogeneic stem cell transplantation					
No	81	49	1		
Yes	43	17	0.53	0.30-0.95	0.034

aSHR, adjusted subhazard ratio; CI: Confidence Interval.

Table S2: Multivariate model for disease-free survival.

	numbers	events	aHR	95% CI	*P* value
Dexamethasone					
No	74	53	1		
Yes	50	20	0.50	0.29-0.84	0.010
AML status					
De novo	109	61	1		
Secondary	15	12	1.93	1.03-3.65	0.041
CD56 (%)					
≤20	90	58	1		
>20	24	9	0.48	0.24-0.97	0.041
LDH – UI/liter					
≤1550	68	35	1		
>1550	56	38	1.73	1.06-2.80	0.027
Hydroxyurea					
No	42	29	1		
Yes	82	44	0.62	0.38-1.03	0.064

aHR, adjusted Hazard Ratio; CI, confidence interval.

Table S3: Multivariate analysis for event-free survival.

	numbers	events	aHR	95% CI	*P* value
Dexamethasone					
No	100	79	1		
Yes	60	30	0.35	0.21-0.58	<0.001
AML status					
De novo	136	88	1		
Secondary	24	21	2.04	1.23-3.39	0.006
Infection at diagnosis					
No	124	78	1		
Yes	33	28	2.29	1.46-3.61	<0.001
LDH – UI/liter					
≤1550	80	47	1		
>1550	80	62	1.51	1.01-2.27	0.046
Fibrinogen- g/liter					
≤ 1.5	15	13	1		
> 1.5	145	96	0.38	0.20-0.71	0.002
Cytogenetic risk – no. (%)					
Favorable	15	5	1		
Intermediate	127	93	2.31	0.92-5.80	0.074
Adverse	18	11	2.49	0.84-7.35	0.098
Hydroxyurea					
No	52	39	1		
Yes	108	70	0.60	0.39-0.93	0.023
Admission in intensive care unit*					
No	114	77	1		
Yes	46	32	2.42	1.49-3.91	<0.001

* during the first three months following chemotherapy; aHR, adjusted Hazard Ratio; CI, confidence interval.

Table S4 : Cytogenetic and molecular characterization of AML cell lines

Name	Sex	FAB	Stage	Karyotype	Model for	*FLT3*		*NPM1*	*IDH1*	*IDH2*	*IDH2*	*DNMT3A*	*CEBPA*	*KIT*	*NRAS*	*KRAS*	*TP53*	*MYC*	*PTEN*
						ITD	TKD		R132	R140	R172								
KG1a	M	M0	Rel	Complex including del 5q, del 7q, -17	Monosomal karyotype; *FGFR1OP2-FGFR1*	wt	wt	wt	wt	wt	wt	wt	wt	wt	mut	wt	mut	overexpressed	mut
HL-60	F	M2	Dx	complex	AML-M2	wt	wt	wt	wt	wt	wt	wt	wt	wt	amp	wt	null; del	amp	wt
Kasumi-1	M	M2	Ref	complex; t(8;21) (q21;q22)	*RUNX1-RUNX1T1*	wt	wt	wt	wt	wt	wt	wt	wt	mut	wt	wt	mut		wt
OCI-AML2	M	M4	Dx	complex; hyperdiploid	*DNMT3A*	wt	wt	wt	wt	wt	wt	R635W	wt	wt	wt	wt	wt		wt
OCI-AML3	M	M4	Dx	complex; hyperdiploid	*NPM1/DNMT3A*	wt	wt	mut	wt	wt	wt	R882C	wt	wt	wt	wt	wt		wt
MV4-11	M	M5	Dx	complex; hyperdiploid; t(4;11)(q21;q23)	*KMT2A-AFF1* /*FLT3*-ITD	ITD	wt	wt	wt	wt	wt	wt		wt	wt	wt	wt	overexpressed	wt
MOLM14	M	M5	Rel	complex; hyperdiploid; t(9;11) (p21;q23)	*KMT2A-MLLT3* /*FLT3*-ITD	ITD	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	overexpressed	wt
U937	M	M5	Ref	complex; t(10;11)(p13;q14)	*PICALM–MLLT10*	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	mut	overexpressed	null

F denotes female; M denotes male; Rel denotes relapse; Dx denotes diagnosis; Ref denotes refractory; wt denotes wild type; Mut denotes mutated; Del denotes deletion/deleted; amp denotes amplification.

Table S5-S7 : see Excel files

Table S5: gene-gene interactions between target genes of residual chemoresistant leukemic cells and those of dexamethasone according to a data-mining algorithm (Genomatix).

Table S6: gene-gene interactions between relapse regulated gene signature and small molecules signatures according to a data-mining algorithm (Genomatix).

Table S7: gene-gene interactions between mutant NPM1 up-regulated gene signature and small molecules signatures according to a data-mining algorithm (Genomatix).

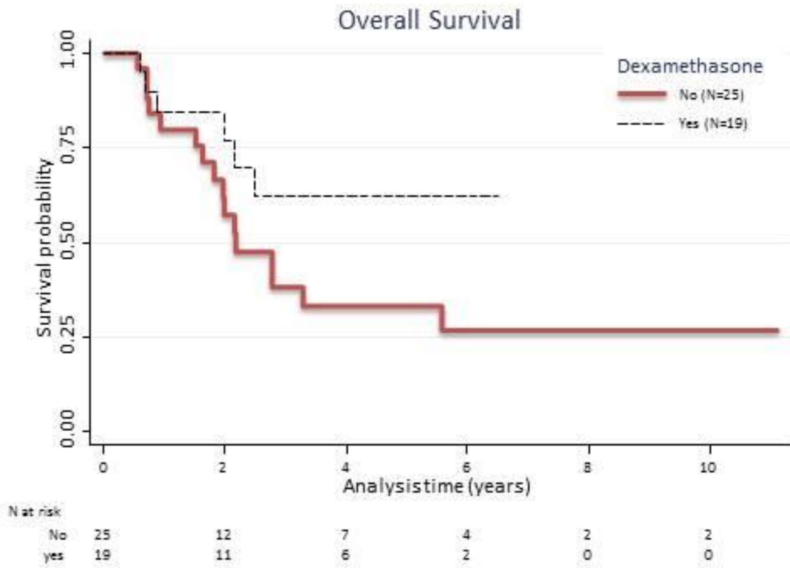
Table S8 : Characteristics of AML samples used for *in vitro* study

Patients	Age	WBC	Karyotype	NPM1	FLT3-ITD
1	47	32,1	46,XY [20]	1	1
2	44	3,2	46, XX [20]	0	0
3	52	59,3	46, XX [20]	1	1
4	85	11,6	46, XY, inv(16)(p13;q22) [20]	not done	not done
5	59	110	46,XY [20]	1	0
6	39	9	46XY, inv(16) [20]	0	0
7	71	5	46,XY [20]	0	0
8	32	215	46, XY, inv(16)(p13;q22) [20]	0	0
9	34	176,3	46XX, inv(16) [20]	0	0
10	78	247	46, XX [20]	0	1
11	75	8,7	46, XX [20]	0	1
12	60	304	46, XX [20]	1	1
13	63	183	46,XY [20]	0	1
14	36	47	47,XY,inv(16)(p13q22) [20],+22 [9]	not done	not done
15	62	10	46,XY [20]	0	1
16	32	136,9	46,XY [20]	1	0
17	64	30	46,XY [20]	0	1
18	57	89	46,XX [20]	0	1
19	44	83,5	46,XY [20]	1	0
20	79	172	46, XY [20]	0	1
21	24	112,8	46,XY [20]	1	1
22	39	39	46,XY [20]	0	1
23	26	25,1	46,XY [20]	0	0
24	68	37,6	46,XY [20]	1	1
25	35	47,8	46, XX [20]	0	1
26	79	19,6	46,XY [20]	0	0
27	57	5,6	46, XX [20]	1	0
28	54	1,91	46, XX [20]	0	0
29	69	0,9	46, XX [20]	0	0
30	51	88	46, XX [20]	1	0
31	61	30	46,XX [20]	1	0
32	73	7,3	46,XY [20]	1	0
33	68	7,7	46, XY [20]	0	1
34	71	68,8	46,XY [20]	1	1
35	78	30,5	46,XY [20]	1	0
36	79	12	46, XX [20]	1	0
37	60	0,86	46,XY [20]	0	0
38	62	93,9	46,XX,inv(4)(p14q24)<6>/ 46,XX,der(21)t(1;21)(q21;q22)<1> 47,XX,der(21)(1;21),+20<3> 46,XX,der(3)t(1;3)(q21;q2?6)<1> 46,XX,der(16)t(1;16)(q21;p13)<1> 46,XX,der(16)t(1;16)(q21;q24)<1>/46,XX<10>	0	0

39	52	342	46,XX [20]	0	0
40	79	247	46,XX [20]	0	1
41	67	95,2	47,XY,+8<11>/46,XY<9>	0	0
42	36	46,1	46,X,t(X;11)(q25;p13)<18>/46,XX<2>	0	0
43	35	207,6	46,XX [20]	0	1
44	79	116	46,XY,t(4;15)(q27;q14)?c<19>/47,idem,+8<1>	1	0
45	64	306	46,XX,[20]	1	0
46	22	162	46,XX [20]	1	0
47	61	304	46,XX [20]	1	1
48	62	146,5	46,XX,t(6;14)(q21;q32)<2>/46,XX<18>	1	1
49	50	114	46,XY [21]	1	1

Supplementary Figures

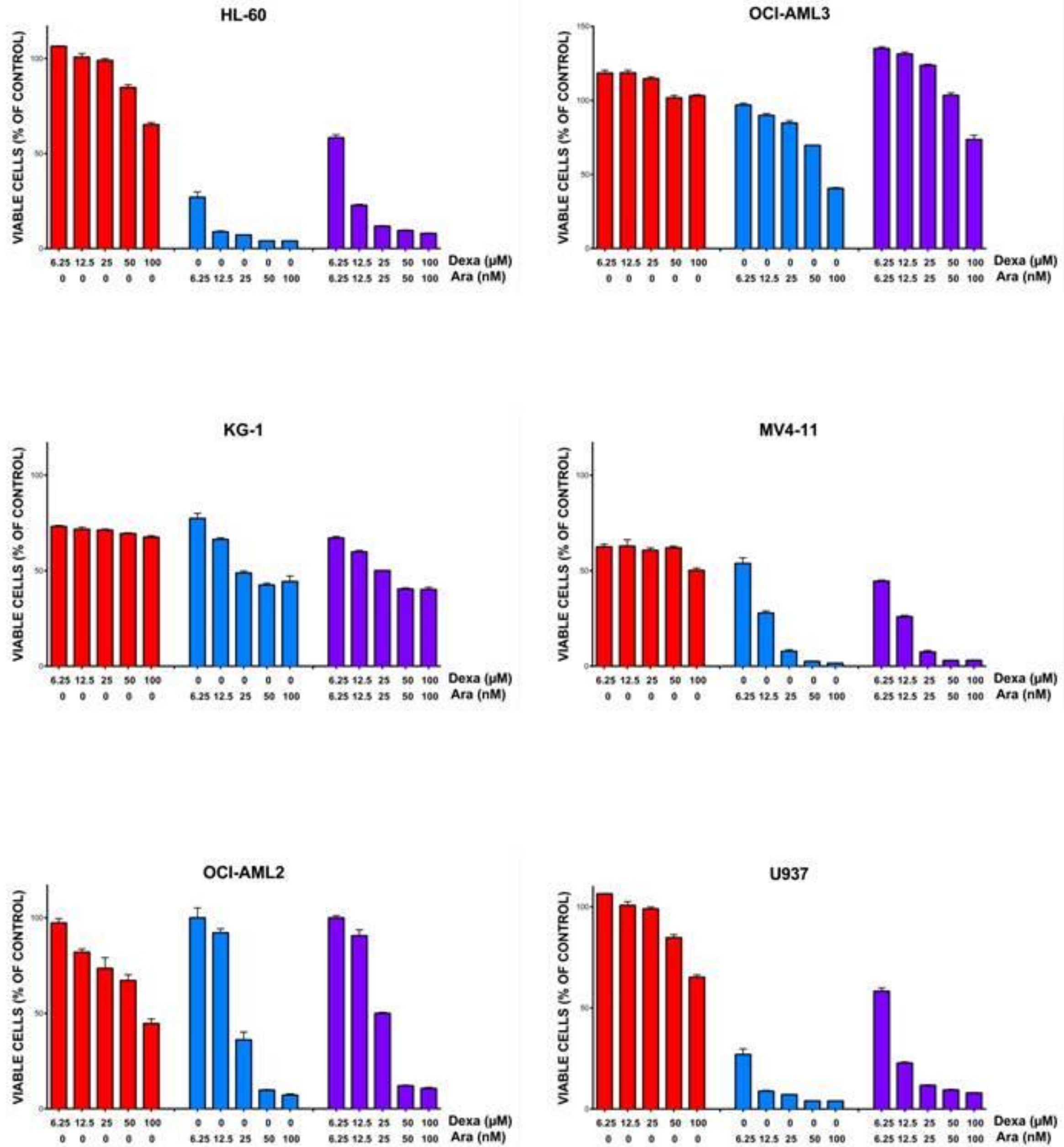
Supplementary Figure 1: OS of patients allografted in CR1 according to dexamethasone treatment



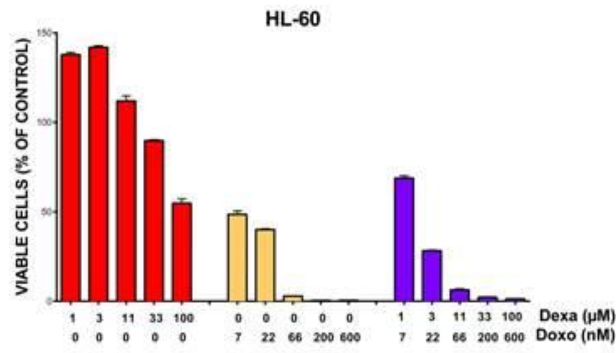
Supplementary Figure 2

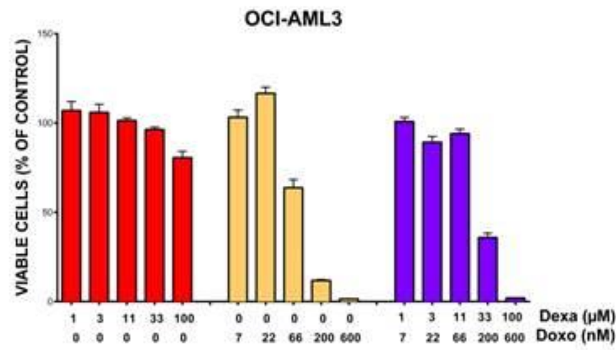
Cell viability in short term liquid cultures. AML cell lines were incubated with dexamethasone, cytarabine (Ara) or dexamethasone plus cytarabine (A) or with dexamethasone, doxorubicin (doxo) or dexamethasone plus doxorubicin (B) for 72 hours in RPMI10% medium and cell viability was assessed using the tetrazolium dye, XTT.

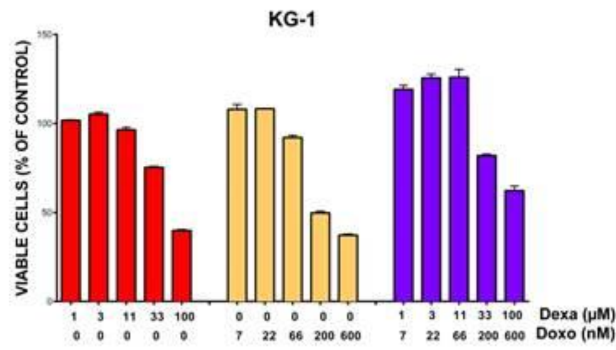
A

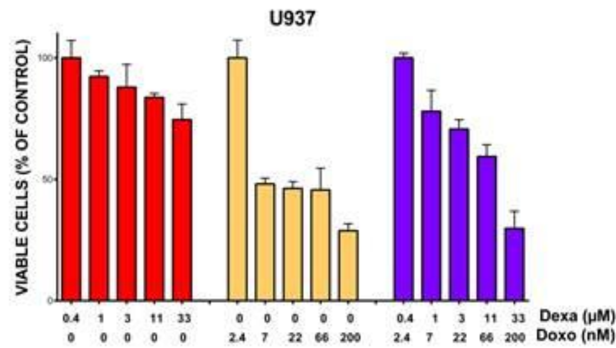


B



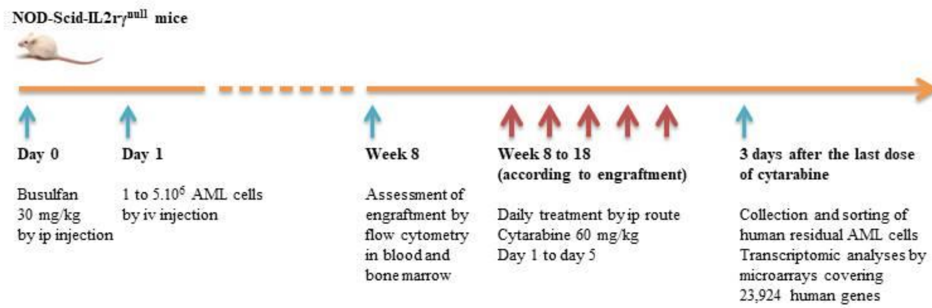




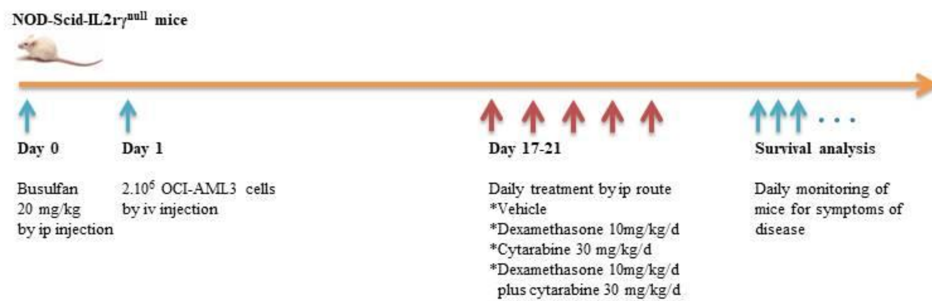


Supplementary Figure 3

A. AML patient-derived xenotransplantation (Farge T et al, Cancer Discovery 2017)



B. OCI-AML3-derived xenotransplantation (Farge T et al, Cancer Discovery 2017)



Supplementary Figure 4

In silico characterization of the putative AML landscape of dexamethasone sensitivity. Transcriptomic data of patients from two data sets (left panel: Verhaak et al.'s data set from 418 patients and in the right panel, TCGA data set from 197 patients) were grouped according to *NPM1* mutational status and other representative subgroups with gene mutations or cytogenetic abnormalities. All genes represented in both panel were mutated. For each group, the false discovery rate q (FDRq) value of enrichment score for dexamethasone gene signature was plotted against normalized enrichment scores (NES). Size of the circle is proportional to the frequency of the population in the corresponding data set. B. Percentages of AML patients according to their molecular characteristics in 2 independent data sets, namely Verhaak et al. (left panel) and TCGA (right panel), and their enrichment for dexamethasone gene signature defined by a positive normalized enrichment score and a FDRq value of <0.25. In the Verhaak et al. data set, molecular subgroups with *NPM1* mutations were significantly enriched in genes responsive to dexamethasone ($P<0{\cdot}001$).