Efficacy of an Fc-modified anti-CD123 antibody (CSL362) combined with chemotherapy in xenograft models of acute myelogenous leukemia in immunodeficient mice

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 doi:10.3324/haematol.2014.113092
Manuscript received on July 2, 2014. Manuscript accepted on April 10, 2015.
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SUPPLEMENTARY MATERIAL

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Supplementary Methods

AML patient samples, xenograft cells, and cell lines

Mononuclear cells were isolated from patient samples by Lymphoprep (Axis-Shield PLC, Dundee, Scotland) or Ficoll (GE Healthcare, Uppsala, Sweden) density gradient separation. AML xenografts serially passaged in mice were harvested by homogenizing the spleen and liver, and mononuclear cells enriched on Lymphoprep density gradients. AML cells were also collected by flushing the bone marrows of engrafted mice. Cells were cryopreserved in liquid nitrogen in heat inactivated fetal calf serum (HIFCS) and 10% DMSO.

In vivo drug treatments

For chemotherapy, mice were treated with 12.5–50 mg/kg daily of AraC (Pfizer, NY, USA) intraperitoneally (IP) for four days and 0.32–1.25 mg/kg of DNR (Pfizer) IV on the fourth day, for a total of 2 or 3 weeks. CSL362 (CSL Limited, Melbourne, Australia) or isotype control was administered IP at 300 μg/dose thrice weekly for 2-6 weeks. In huNK adoptive transfer experiments, 5–20 x 10^6 huNK cells were IV inoculated per mouse. Ten or 500 IU of human IL-2 (Peprotech, NJ, USA) per mouse were administered IP with each transfer of NK cells.

An event was defined *a priori* to occur when the hCD45+ reached 25% in the blood or when mice exhibited signs of leukemia related morbidity. Efficacy was expressed as leukemia growth delay (LGD), defined as the difference in median event-free survival (EFS) between the treated and control groups. Mice were also sacrificed less than 5 days after the last treatment to assess leukemia burden in bone marrow, spleen, blood, and liver.

Cell staining and flow cytometry

Cells were stained with fluorescence conjugated anti-mouse antibodies to CD45 and anti-human antibodies to CD3, CD16, CD33, CD34, CD38, CD45, CD56, CD123 (clone 9F5 or 7G3), NKp46 and 7-AAD (BD Biosciences or Biolegend, CA, USA). Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest Pro v6.0 (BD Biosciences) and Cyflogic v1.2.1 (CyFlo Ltd.) software. A known number of counting beads (Beckman Coulter) was added to specific volumes of blood, or cells collected from single femurs to facilitate absolute counts of mononuclear cells. The relative fluorescence intensity (RFI) was calculated as the ratio of the geometric mean of cells stained with antibody to isotype control. CSL362 and clone 7G3 bind the same epitope while clone 9F5 binds an independent epitope on CD123 (data not shown). CSL362 binding and surface CD123 expression can be determined by staining with both antibody clones on control and CSL362 treated cells. In some experiments AML

or NK cells were stained with 2 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) at room temperature for 5 minutes to distinguish them from each other.

Mutation analyses of AML xenografts

MLL-PTD was quantified using primers (Forward 5'-GTCCAGAGCAGAGCAAACAGAA-3' and Reverse 5'-GAAAACACAGATGGATCTGAGAGG-3'; Geneworks Pty Ltd, Hindmarsh, Australia) and probe (5'-6FAM-CTCCCCGCCCAAGTA-MGBNFQ-3'; Applied Biosystems) and *ABL1* quantified using an inventoried *ABL1* Taqman assay (HS01104728_M1; Applied Biosystems). *MLL*-PTD cDNA for standard curve derivation was obtained from EOL-1 cells (gifted by Lois A Salamonsen, Prince Henry's Institute of Medical Research, Melbourne, Australia). Amplified *MLL* exon 9/3 PTD cDNA was cloned into the pGem-T vector, purified and diluted. An *ABL1* cDNA clone (Origene, MD, USA) was used to generate *ABL1* standards of appropriate concentration. Total RNA from xenograft recipients was DNase treated using Ambion Turbo DNA free and cDNA synthesized using Invitrogen Superscript III (Life Technologies). Based on a reference range we established from the blood or bone marrow of 23 controls, *MLL*-PTD was considered to be positive if the *MLL*-PTD:*ABL1* was $\geq 10 \times 10^{-3}$.

Isolation and expansion of huNK cells and ADCC assay

HuNK cells were co-cultured with irradiated SMI-LCLs (100 Gy), at a 1:20 ratio in 96-well U-bottom plates in X-vivo 20 media (Lonza, Basel, Switzerland) supplemented with 2 mM Glutamax-1, 500 IU/mL human IL-2 (Peprotech), and 10% HIFCS. The cells were sub-cultured on day 7, 9, 12 and 15. Cultures >15 days were fed by replacing half the media with fresh media weekly. Some NK cells were expanded in co-culture with K562-mIL15-41BBL as previously described (Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T*, et al.* Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res* 2009; **69**(9)**:** 4010-4017).

In ADCC assays, expanded huNK cells were co-cultured with CFSE+ AML cells at a 10:1 ratio in 96-well U-bottom plates in RPMI1640 supplemented with 80 IU/ml human IL-2, 10% HIFCS with 10 μg/ml of isotype control or CSL362 antibody. The plates were incubated at 37°C in 5% CO_2 for 5 hours. Specific cell lysis was quantified by exclusion of 7-AAD and flow cytometry.

Statistical analyses

The Mann-Whitney non-parametric two-tailed test was used to compare differences between two groups. Kaplan-Meier survival curves were compared by logrank test. Gradients were compared with linear regression analyses, and Spearman's rank correlations were used. A paired *t*-

test was performed to compare the changes in immunophenotype of AML cells harvested from different organs of an individual mouse. All data were analyzed using Prism 5/6 software (GraphPad Software, CA, USA).

Supplementary Tables

Supplementary Table S1. Median event free survival of immunodeficient mice engrafted with human AML xenografts.

The rate of engraftment for each mouse was quantified based on the event free survival, defined as the time in number of days post-transplantation for the human leukemia engraftment to reach 25% in the blood. The event free survival significantly decreased with subsequent passages for AML-4 ($P = 0.0004$), AML-5 ($P = 0.0003$), AML-16 ($P = 0.0002$), AML-17 ($P = 0.0014$) and AML-18 ($P = 0.0055$), but not for AML-9 ($P = 0.16$), based on logrank test for trend in survival (data not shown). This data is represented graphically in Supplementary Figure S2.

AML xenografts took longer to reach events in NOD/SCID mice when compared with NSG mice (Supplementary Figure S3). Surprisingly, continuous AML xenografts engrafted faster in NSG mice which have not been subjected to sub-lethal irradiation compared with irradiated mice (Figure 2B).

AML sample	Passage number						Mouse strain		Irradiated	Not irradiated
	1º	2º	3º	4º	5º	6º	NSG	NOD/ SCID		
4	253	112	NA	NA	NA	NA	NA	NA	112	86
5[&]	>48[1]	62	55	49	45	41	NA	NA	63[&]	120[&]
9	95	59	87	NA	NA	NA	58	162	NA	NA
16	NA[2]	78	39	57	40	NA	NA	NA	51	44
17	>163	107	89	88	NA	NA	75	92	165	148
18	~30^	44	29	29	28	NA	22	43	NA	NA

[1]Mice were sacrificed on day 48 before AML-5 had the opportunity to further expand in blood
[2]AML-16 was expanded in NOD/SCID mice in the first passage while the other passages were expanded in NSG mice, hence the first passage data was excluded from this table.
[&]AML-5 was expanded in NOD/SCID mice while all the other AMLs were expanded in NSG mice.
^Approximated from the fixed time point sacrifices of 3 mice.
NA = not available.

Supplementary Table S2. Fold increase of human NK cells expanded *ex vivo* over 25 days.

Primary human NK cells isolated from primary human peripheral blood mononuclear cells were cultured with gamma-irradiated SMI-LCL feeder cells at a ratio of 1:20 in 96-U-well plates. The cultures were split over two plates and media added on days 7, 9, 12 and 15. Subsequently, cultures were fed every 7 days by replacing half of the culture supernatant with fresh media.

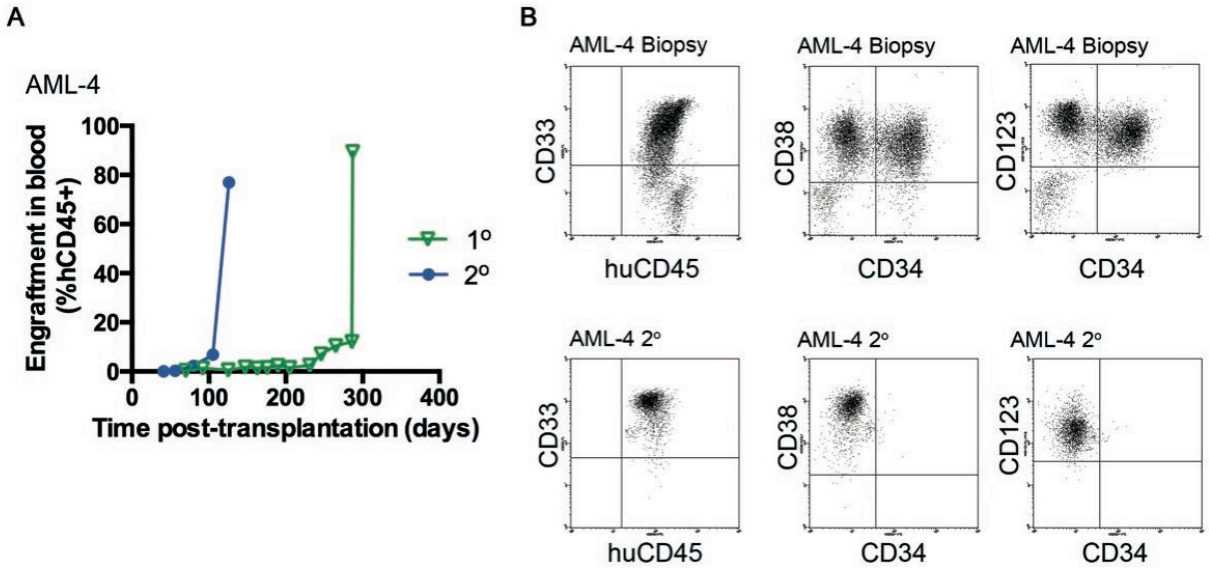
NK donor	Fold increase
Donor 51691 n=2	669 (avg.)
Donor 70382 n=2	622 (avg.)
Donor 47008 n=1	725

Supplementary Table S3. Event free survival and leukemia growth delays of mice treated with CSL362 and/or NK cells, and/or AraC/DNR.

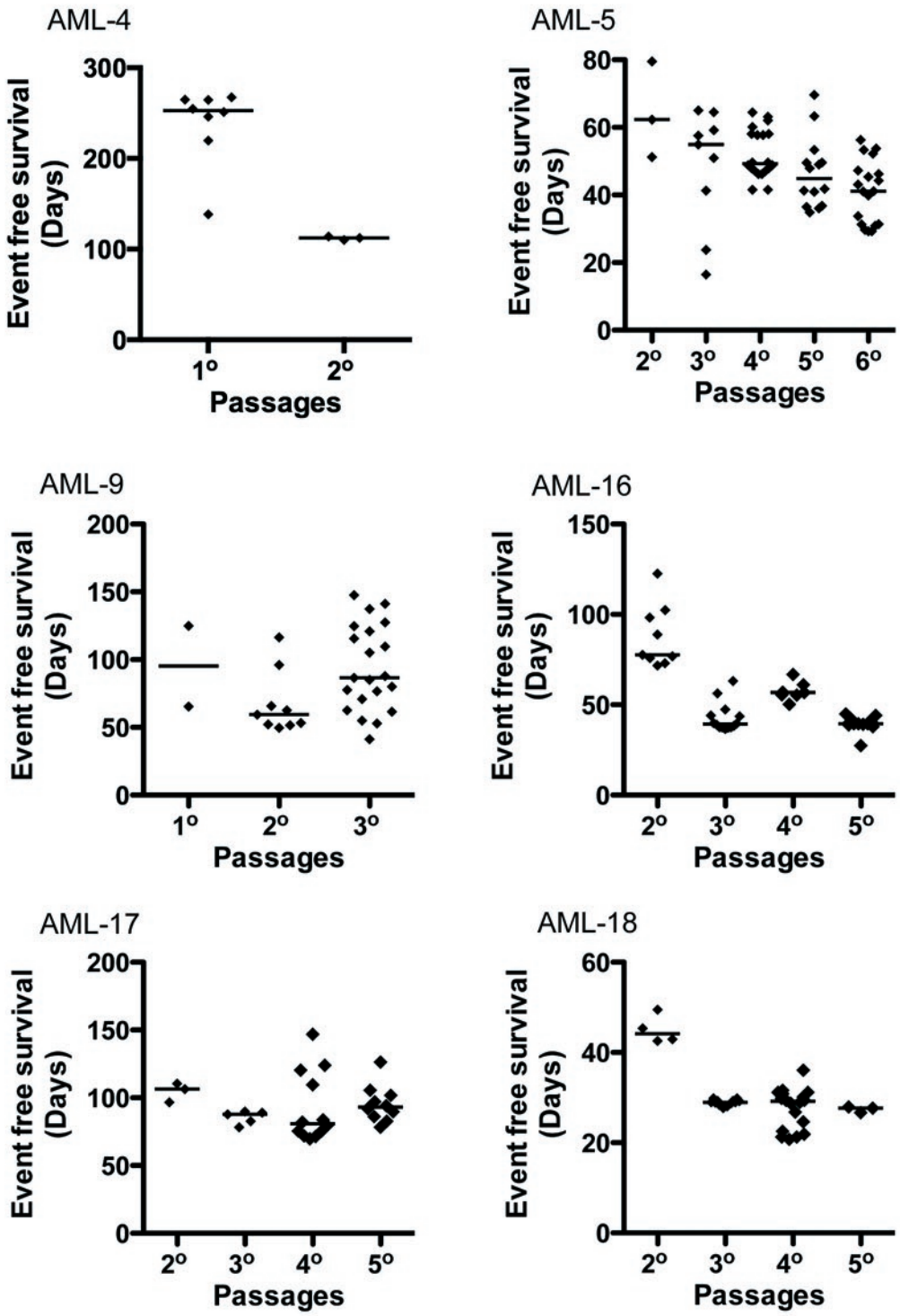
Xenograft	Treatments	EFS (days)	LGD (days)	Significance
AML-5 (10IU IL-2 injected with NK cells) Fig. S11A, B	IgG1	3.0		
	IgG1 + 4.6 x 10^6 NK	5.0	2.0 (vs. IgG1)	0.0016** (vs. IgG1)
	CSL362	4.1	1.1 (vs. IgG1), -0.9 (vs. IgG1+NK)	0.048* (vs. IgG1), n.s. (vs. IgG1+NK)
	CSL362 + 4.6 x 10^6 NK	5.3	2.3 (vs. IgG1), 1.2 (vs. IgG1+NK), 0.3 (vs. CSL362)	0.003** (vs. IgG1), n.s. (vs. IgG1+NK), n.s. (vs. CSL362)
AML-5 (no IL-2) Fig. S11C, D	IgG1	19.0		
	IgG1 + 10 x 10^6 NK	10.7	-8.3 (vs. IgG1)	n.s. (vs. IgG1)
	CSL362	15.6	-3.4 (vs. IgG1), 4.9 (vs. IgG1+NK),	n.s. (vs. IgG1), n.s. (vs. IgG1+NK)
	CSL362 + 10 x 10^6 NK	20.1	1.1 (vs. IgG1), 9.4 (vs. IgG1+NK), 4.5 (vs. CSL362)	n.s. (vs. IgG1), n.s. (vs. IgG1+NK), n.s. (vs. CSL362)
AML-16 (500IU IL-2 injected with NK cells) Fig. 6 A-D	IgG1	56.8		
	IgG1 + 20 x 10^6 NK (x2)	58.3	1.5 (vs. IgG1)	n.s. (vs. IgG1)
	CSL362	62.1	5.3 (vs. IgG1), 3.8 (vs. IgG1+NK),	n.s. (vs. IgG1), 0.0077** (vs. IgG1+NK)
	CSL362 + 20 x 10^6 NK (x2)	59.3	2.5 (vs. IgG1), 1.0 (vs. IgG1+NK), -2.8 (vs. CSL362)	n.s. (vs. IgG1), n.s. (vs. IgG1+NK), n.s. (vs. CSL362)
AML-16 (500IU IL-2 injected with NK cells) Fig. 6 E-H	Chemo + IgG1	61.0		
	Chemo + IgG1 + 20 x 10^6 NK (x2)	61.3	0.3 (vs. chemo+IgG1)	n.s. (vs. chemo+IgG1)
	Chemo + CSL362	66.9	5.9 (vs. chemo+IgG1), 5.6 (vs. chemo+IgG1+NK)	0.02* (vs. chemo+IgG1), 0.002** (vs. chemo+IgG1+NK)
	Chemo + CSL362 + 20 x 10^6 NK (x2)	76.2	15.2 (vs. chemo+IgG1), 14.9 (vs. chemo+IgG1+NK), 9.3 (vs. chemo+CSL362)	0.0004*** (vs. chemo+IgG1), 0.0004*** (vs. chemo+IgG1+NK), 0.0008*** (vs. chemo+CSL362)
AML-17 (NOD/SCID, no IL-2) Fig. S11E, F	IgG1	96.6		
	IgG1 + 5 x 10^6 NK (x2)	93.6	-3.0 (vs. IgG1)	n.s. (vs. IgG1)
	CSL362	100	3.4 (vs. IgG1), 6.4 (vs. IgG1+NK)	n.s. (vs. IgG1), n.s. (vs. IgG1+NK)
	CSL362 + 5 x 10^6 NK (x2)	108	11.4 (vs. IgG1), 14.4 (vs. IgG1+NK), 8.0 (vs. CSL362)	n.s. (vs. IgG1), n.s. (vs. IgG1+NK), n.s. (vs. CSL362)
AML-17 (500IU IL-2 injected with NK cells) Fig. S13 A-D	Chemo + IgG1	82.7		
	Chemo + IgG1 + 6 x 10^6 NK (x6)	75.8	-6.9 (vs. IgG1)	n.s. (vs. chemo+IgG1)
	Chemo + CSL362	90.8	8.1 (vs. chemo+IgG1), 15 (vs. chemo+IgG1+NK)	0.03* (vs. chemo+IgG1), 0.006** (vs. chemo+IgG1+NK)
	Chemo + CSL362 + 6 x 10^6 NK (x6)	80.6	-2.1 (vs. chemo+IgG1), 4.8 (vs. chemo+IgG1+NK), -10.2 (vs. chemo+CSL362)	n.s. (vs. chemo+IgG1), n.s. (vs. chemo+IgG1+NK), 0.02* (vs. chemo+CSL362)

n.s. = non-significant

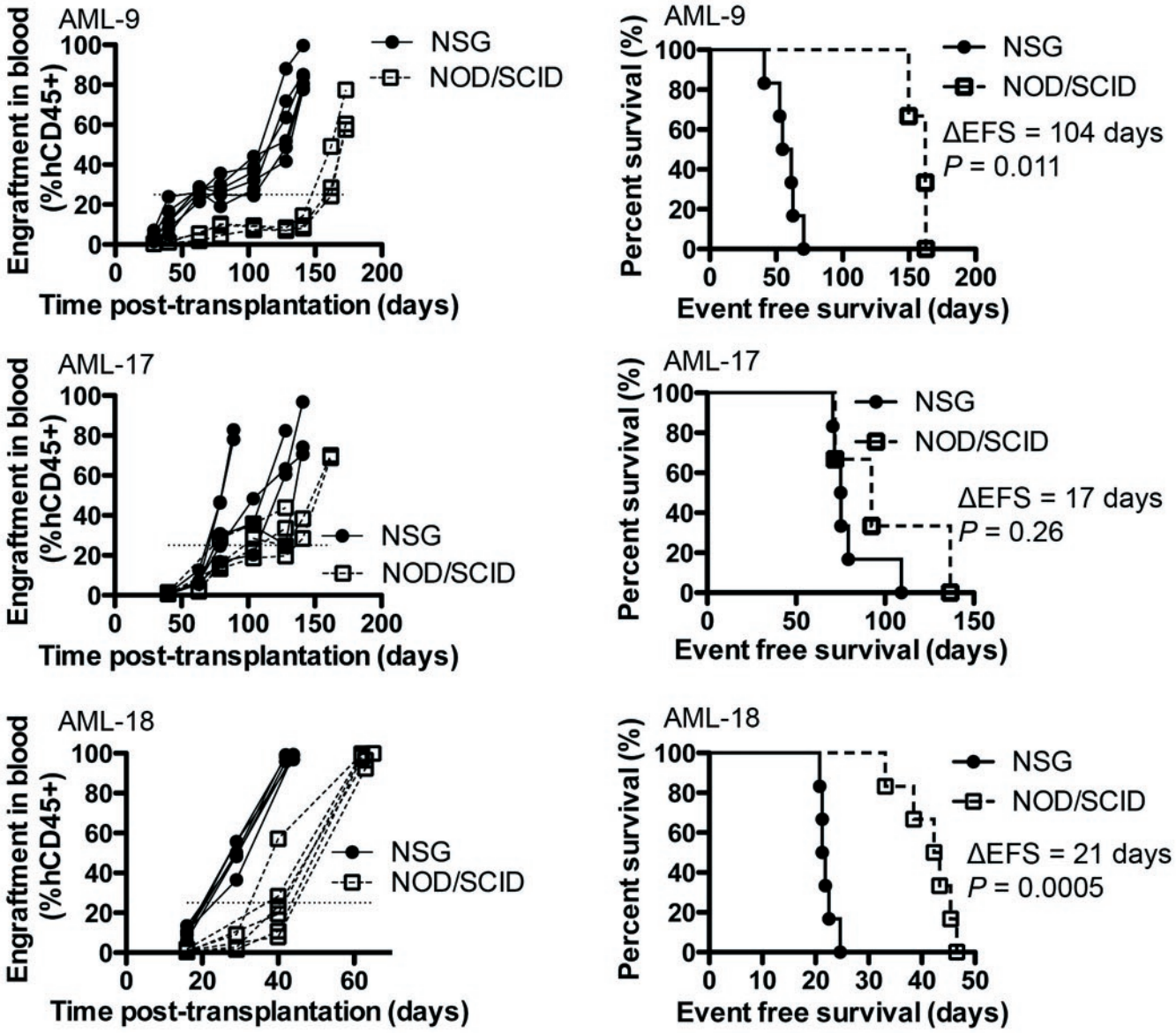
Supplementary Figures



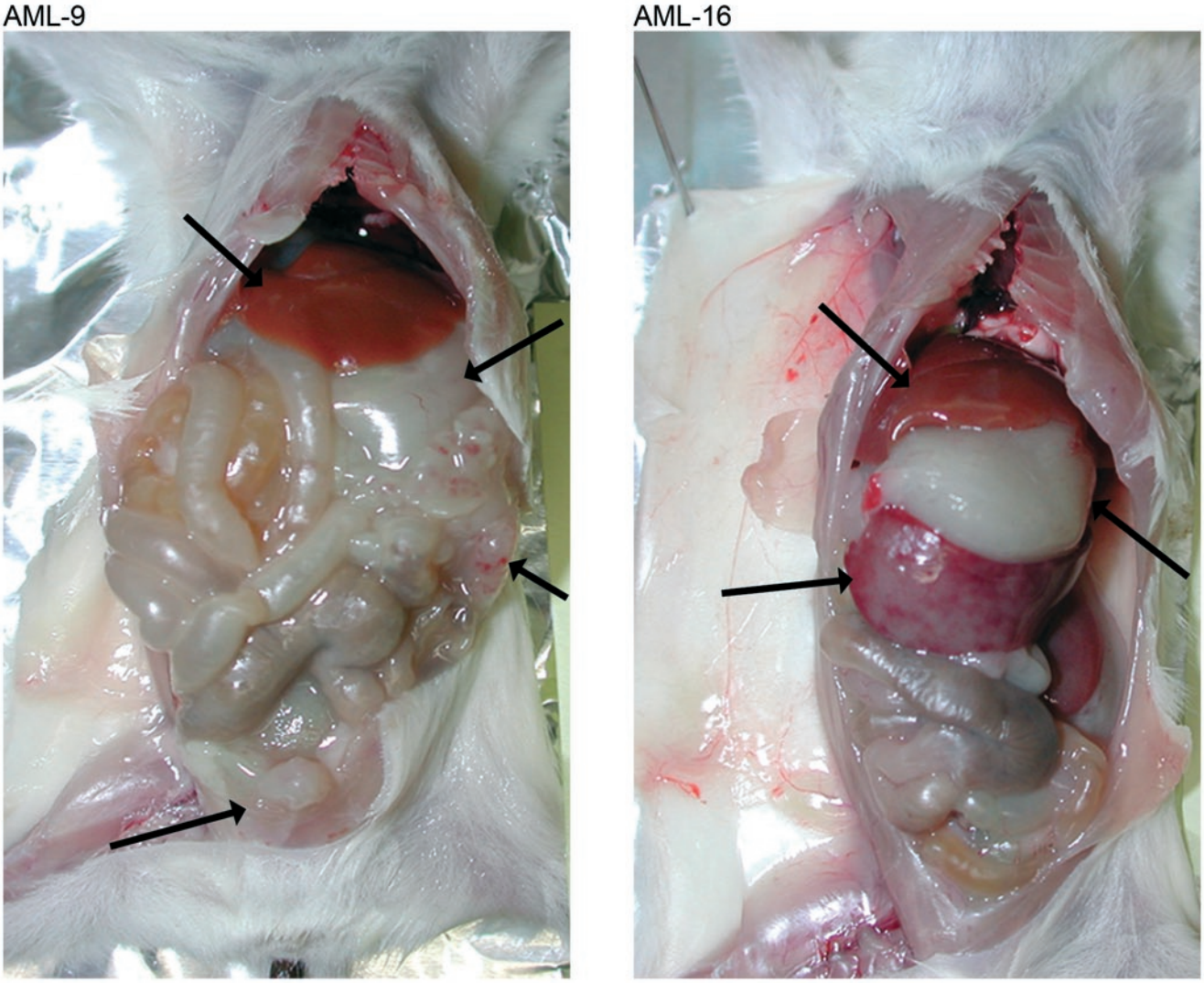
Supplementary Figure S1. AML-4 engraftment in the blood and immunophenotypes in NSG mice. (A) The engraftment of different passages of AML-4 in the blood over time post-transplantation. Each line represents the median engraftment level for an individual experiment with n = 3-8 mice per experiment. (B) Representative scatter plots of immunophenotype of the original patient AML-4 sample and second passage xenograft cells harvested from mouse spleens, based on the expression of CD34, CD38 and CD123, gated on human CD45+ cells. Quadrant lines were based on fluorescence levels of corresponding isotype controls.



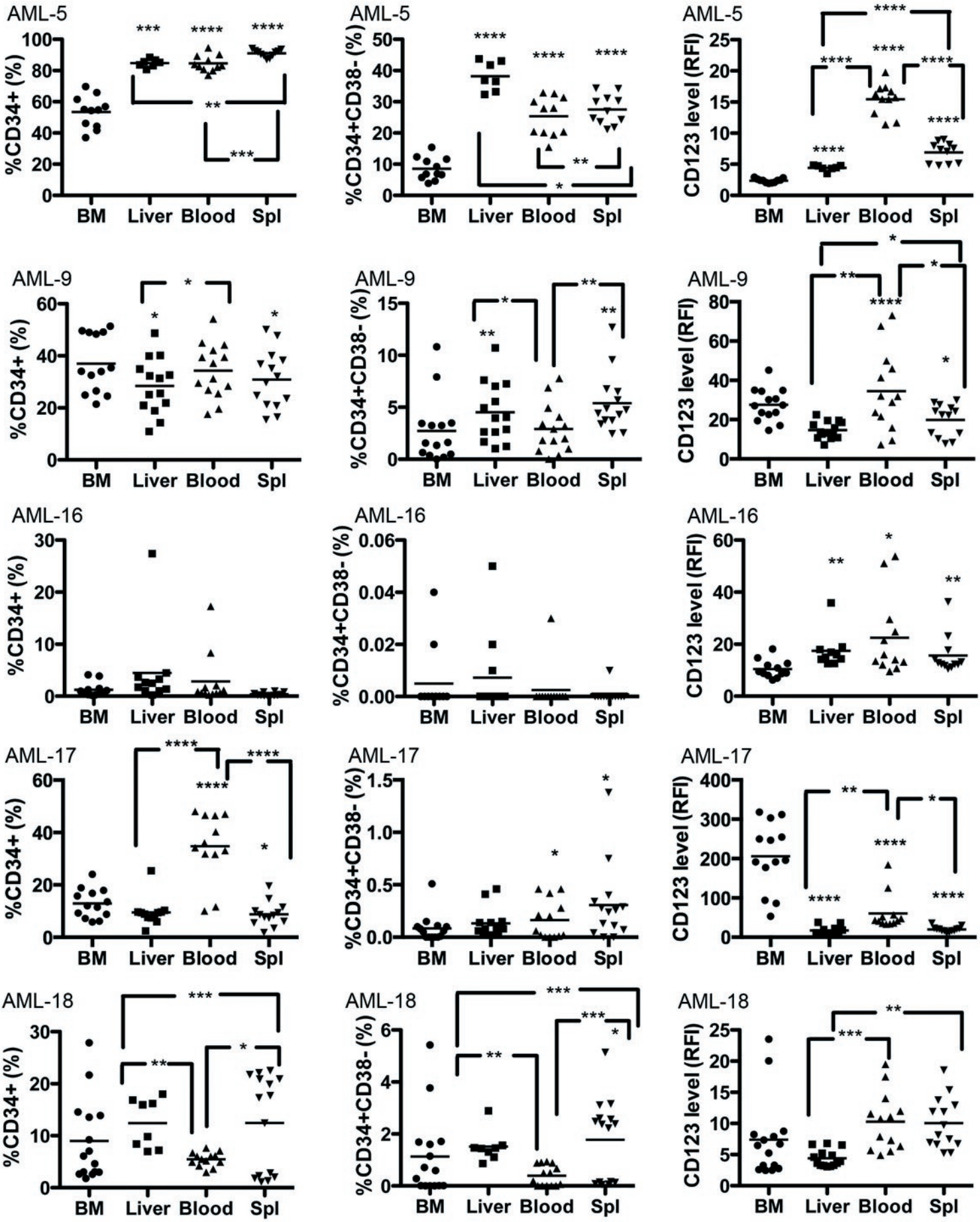
Supplementary Figure S2. EFS of mice engrafted with increasing passages of different AML xenografts. EFS was defined as the time in number of days post-transplantation for the human leukemia engraftment to reach 25% in the blood. Each individual point represents data from a single mouse. Horizontal bars represent median values. For each passage, data was pooled from 1-4 independent experiments.



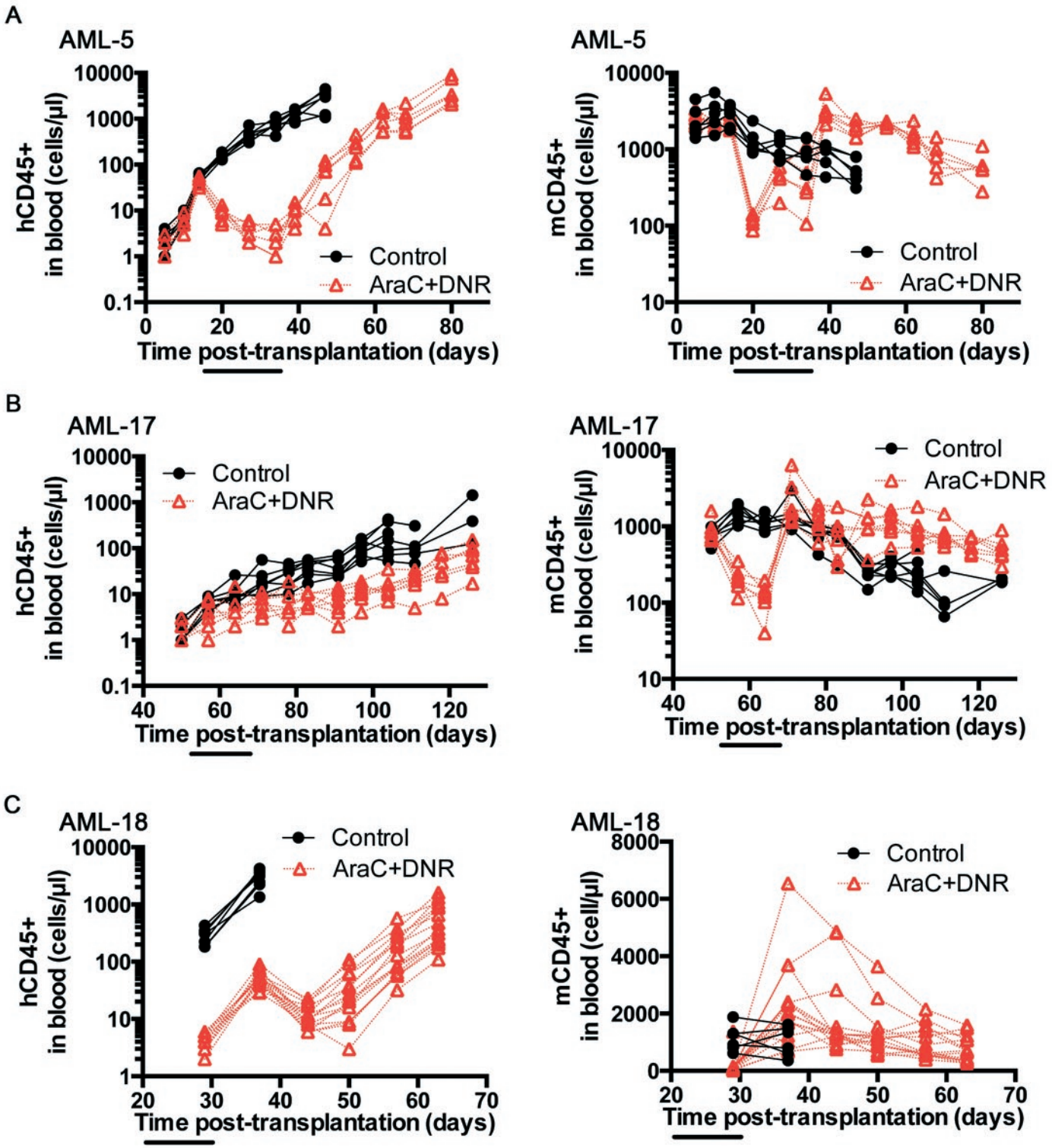
Supplementary Figure S3. Comparison of AML xenograft engraftment rates in NOD/SCID and NSG mice. Engraftment levels of AML cells in the blood over time in NSG and NOD/SCID mice (left) and respective EFS curves (right). Each line represents the engraftment level in the blood of an individual mouse at different time points, n = 3-6 mice per group.



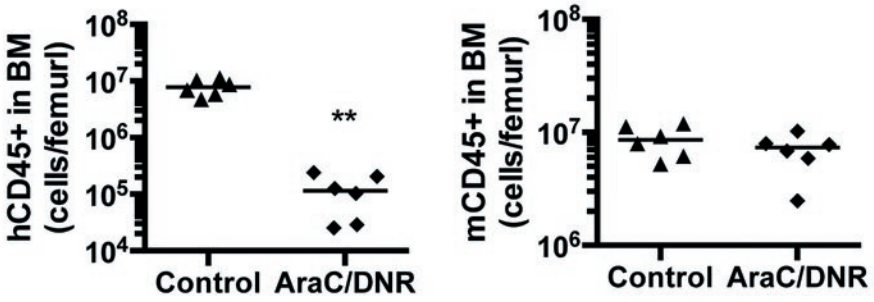
Supplementary Figure S4. Sample autopsy pictures of AML-9 and AML-16 engrafted mice. AML-9 and AML-16 xenografts produced distinctive anatomical changes at the final stages of the disease. Arrows highlight specific tumor growth sites, stomach enlargement, hepatomegaly and splenomegaly.



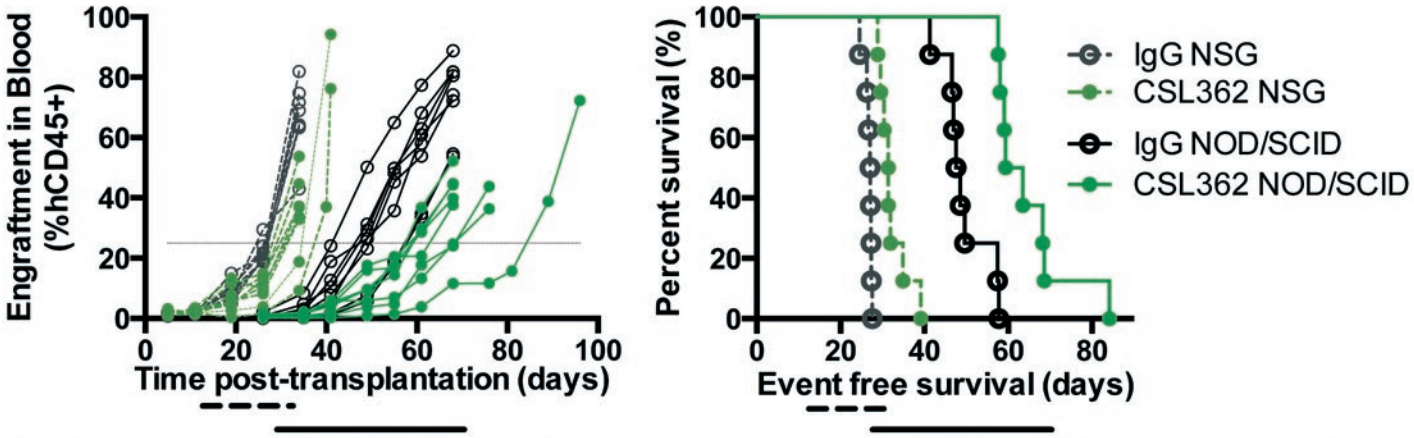
Supplementary Figure S5. Immunophenotypes of AML cells in different engrafted mouse organs based on %CD34+, %CD34+CD38-, and surface CD123 expression. Each individual point represents data from a single mouse, data were pooled from one to three experiments for each xenograft with n = 3-12 mice per experiment. Paired t test was used, and horizontal bars represent means. Asterisks (*) above specific groups represent significant difference when compared against bone marrow (BM). Spl = Spleen. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; and **** = $P < 0.0001$.



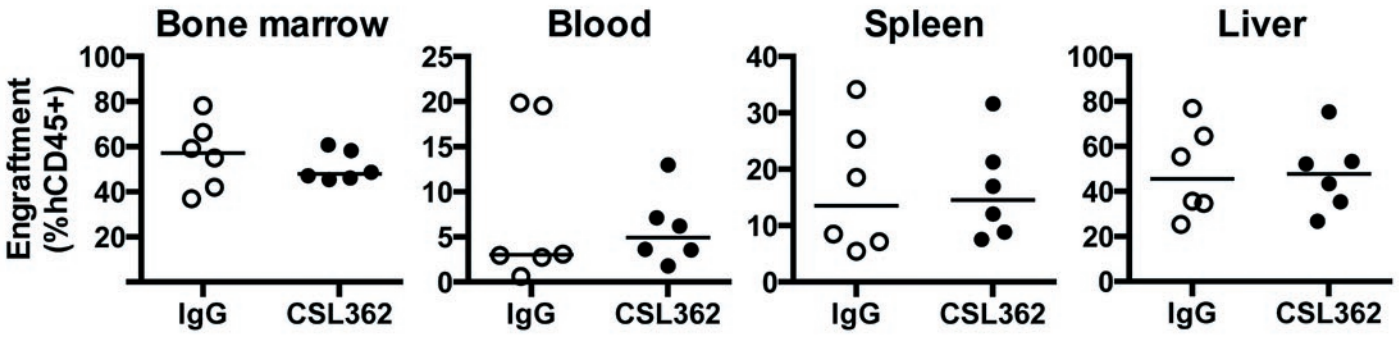
Supplementary Figure S6. AraC and DNR treatments reduced absolute AML blast and normal hematopoietic cell counts. Absolute AML blast (left) and mouse hematopoietic cell count (right) in blood for (A) AML-5, (B) AML-17, and (C) AML-18 engrafted NOD/SCID (AML-17) and NSG (AML-5 and 18) mice treated with 2-3 weeks of AraC (25 mg/kg) and DNR (0.63 mg/kg). Horizontal bars underneath the graphs represent time of treatment, each line represents results from an individual mouse (n = 6-15 mice per group). The %hCD45+ engraftment and EFS curves corresponding to these absolute count data are in Figure 3.



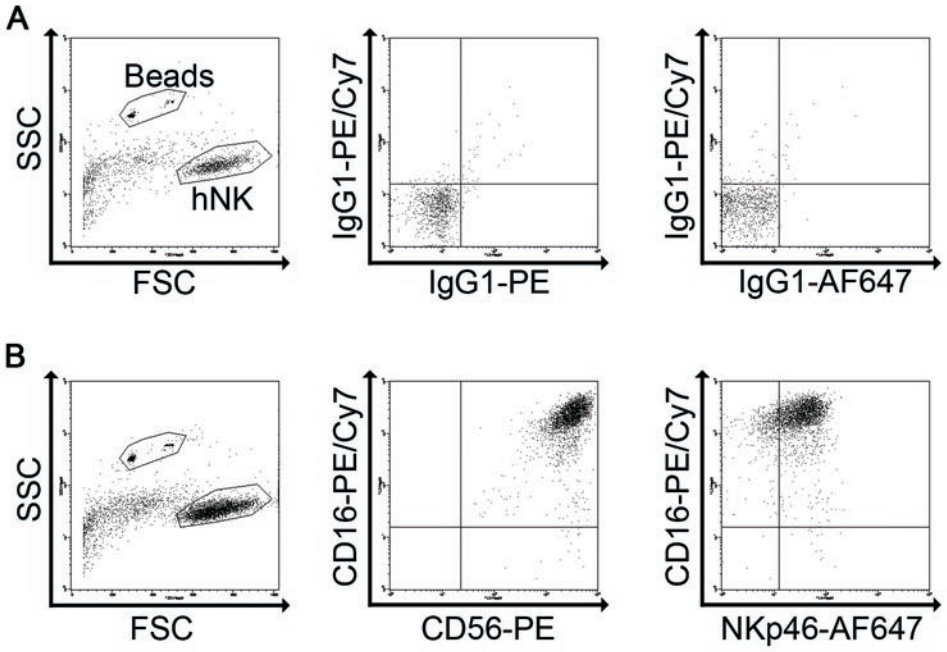
Supplementary Figure S7. AraC and DNR treatments reduced absolute AML-5 blast but not normal hematopoietic cell counts in the bone marrow. Absolute AML blast (left) and mouse hematopoietic cell (right) counts in the bone marrow of AML-5 engrafted NSG mice after 3 weeks of AraC (25 mg/kg) and DNR (0.63 mg/kg) treatments, which were initiated at 14 days post-transplantation. Each point represents an individual mouse (n = 6 mice per group) and the horizontal line represents the median. The %hCD45+ engraftment graph corresponding to these absolute count data is in Figure 4A. ** = $P < 0.01$.



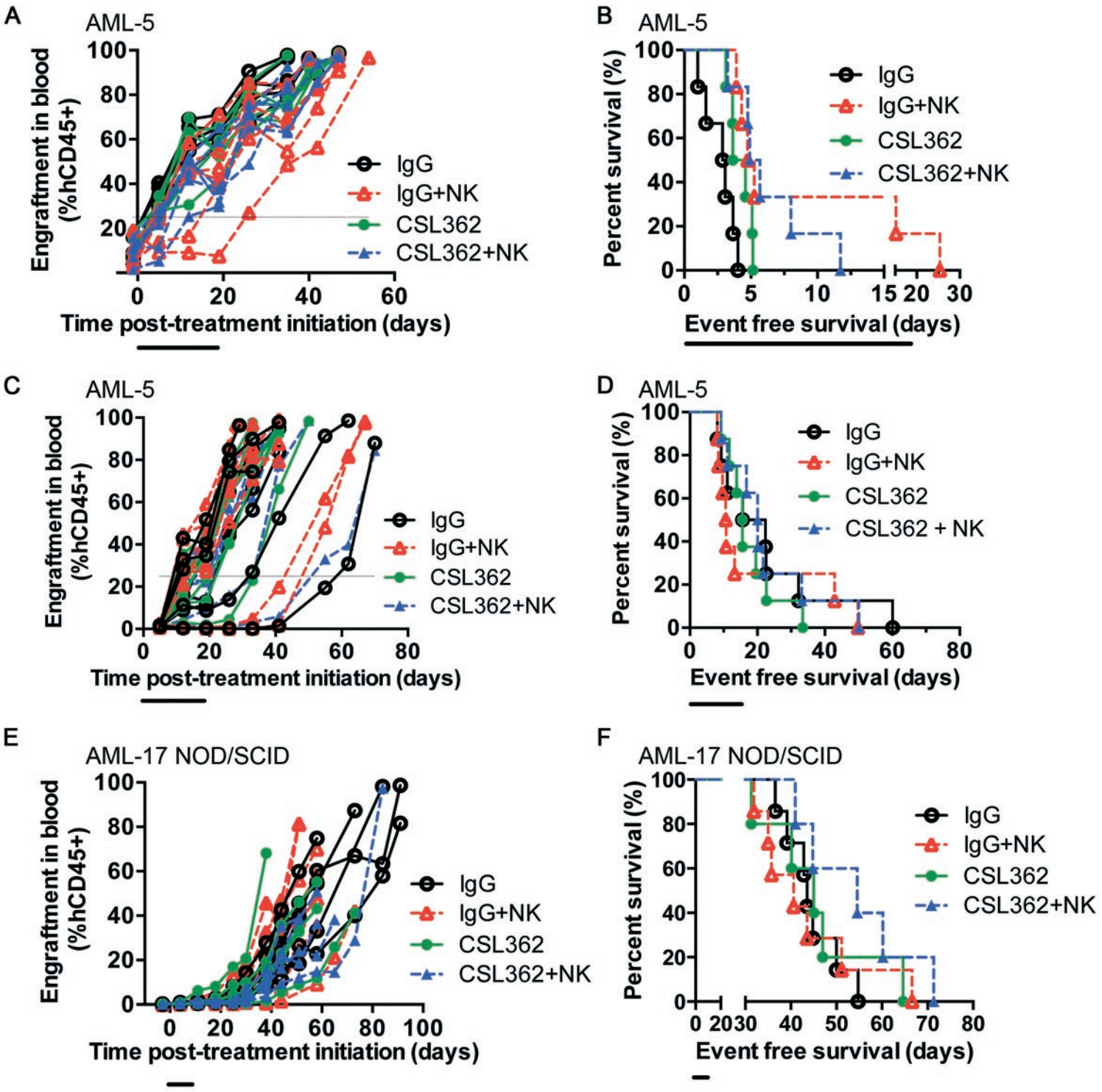
Supplementary Figure S8. Efficacy of CSL362 in AML-5 engrafted NSG and NOD/SCID mice. Engraftment levels in blood (left) and EFS curves (right), where an event was predefined as 25% hCD45+ cells in the blood, are shown. CSL362 treatments were initiated when the %hCD45+ in blood reached 1%. The dashed and solid black bars beneath the graphs represent treatment periods for NSG and NOD/SCID mice, respectively. The NSG groups received 3 weeks of treatment compared with 6 weeks in NOD/SCID mice because all the NSG mice reached events within the 3 week treatment period. The LGD for CSL362 treated NSG and NOD/SCID mice were 4 and 13 days, respectively ($P < 0.0001$ when compared with respective isotype controls, n = 8 mice per group).



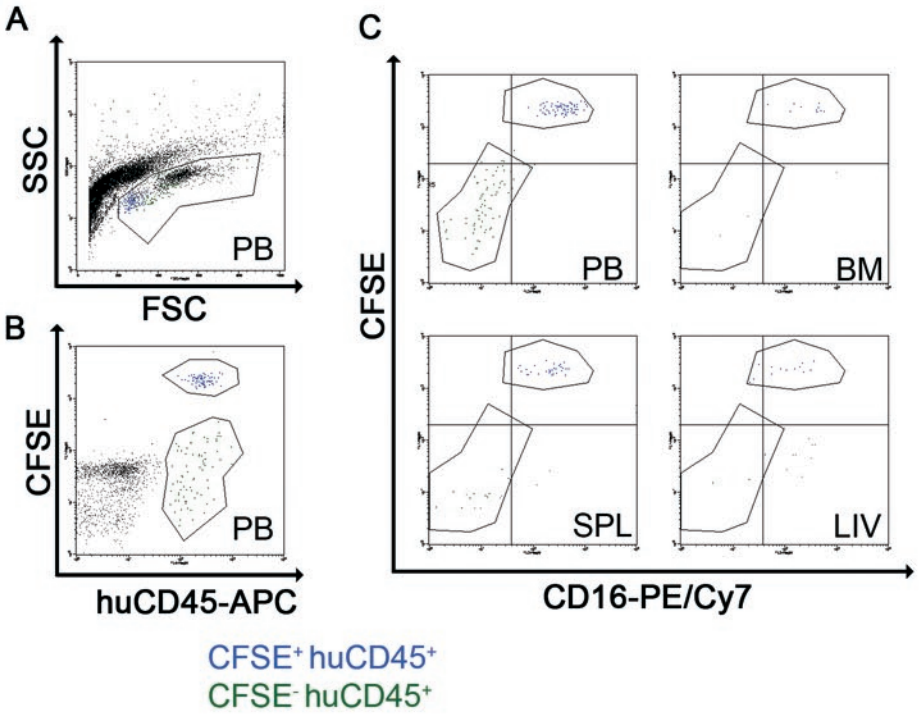
Supplementary Figure S9. Efficacy of CSL362 in various organs of AML-18 engrafted NOD/SCID mice. Engraftment levels of AML-18 in bone marrow, blood, spleen and liver of mice treated with 3 weeks of CSL362 (300µg, thrice weekly IP) or isotype control initiating 6 days post-transplantation. Each point represents an individual mouse (n = 6 mice per group) and the horizontal line represents the median.



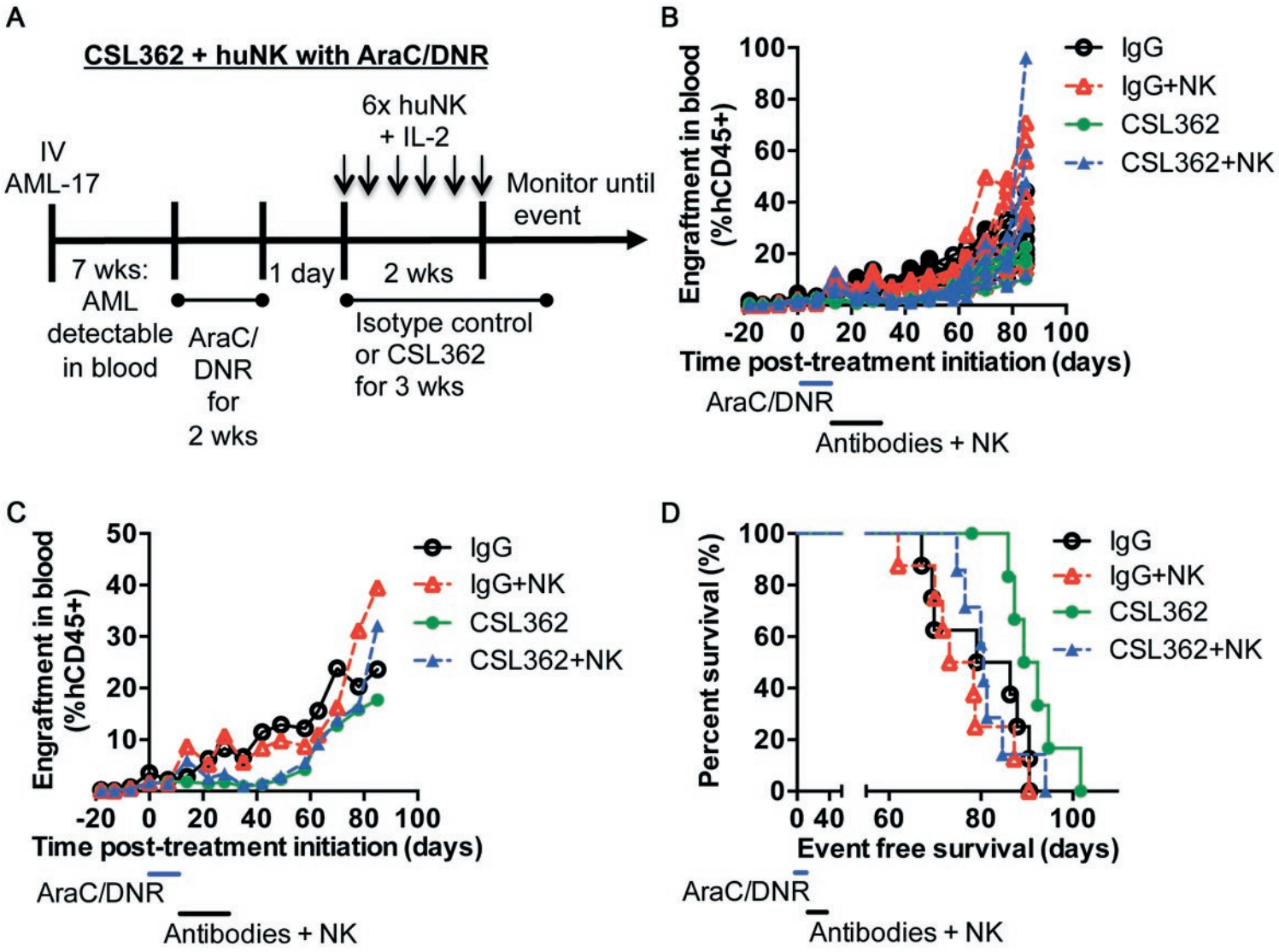
Supplementary Figure S10. Representative immunophenotype of huNK cells expanded for 21 days. (A) Scatter plots of huNK cells stained with isotype controls, which set the quadrant lines. (B) Scatter plots gated for live mononuclear cells showed huNK cells with high levels of surface expression of CD16, and CD56, in addition to expression of NKp46, consistent with immunophenotypes of active huNK cells. The "Beads" gate encompassed counting beads.



Supplementary Figure S11. In vivo efficacy of CSL362 and adoptively transferred huNK cells against AML xenografts in immunodeficient mice. In all three experiments, treatments with antibodies and varying doses of huNK cells commenced when leukemia cells were detectable in the blood. (A, C, E) AML engraftment levels in the blood of individual mice over time and (B, D, F) corresponding EFS curves are shown. (A, B) AML-5 engrafted NSG mice received 3 weeks of antibody treatment, and a single injection of 4.6 million NK cells and 10 IU of IL-2 with the first dose of antibodies. CSL362, NK cells and the combination produced small but significant LGD against controls ($P < 0.05$, n = 6 mice per group, Supplementary Table S3), but this was not repeated in a subsequent experiment (C, D). (C, D) AML-5 engrafted NSG mice received 3 weeks of antibody treatment, and a single injection of 10 million NK cells without IL-2 with the first dose of antibodies. (E, F) AML-17 engrafted NOD/SCID mice received 2 weeks of antibody treatment and 2 weekly injections of 5 x 10^6 NK cells each with the first dose of antibodies each week. Overall there was no significant difference in AML progression between any groups (Supplementary Table S3, n = 6 - 8 mice per group).



Supplementary Figure S12. Migration of huNK cells to various mouse organs. Representative scatter plots of different homogenized tissues from AML-16 engrafted NSG mice injected with expanded CFSE labeled huNK cells 24 hours prior. (A) Peripheral blood (PB) sample demonstrating the clear presence of CFSE+hCD45+ huNK cells in blue and a small but detectable number of CFSE-hCD45+ leukemia cells in green on the forward and side scatter plot. (B) Scatter plot gated on hCD45+ cells highlighted the distinction between CFSE+ huNK cells and CFSE- leukemia cells. (C) Representative scatter plots of peripheral blood, bone marrow, spleen and liver samples demonstrating the presence of CFSE+ huNK cells, which expressed high levels of CD16.



Supplementary Figure S13. Efficacy of CSL362 with or without adoptive transfer of huNK cells after chemotherapy against AML-17 in NSG mice. (A) Regimen schematic describing treatment of AML-17 engrafted mice with CSL362 and adoptive transfer of huNK cells after chemotherapy. Six million huNK cells were transplanted with 500IU of IL-2 per injection, thrice per week, delivered with the antibodies each day for two weeks. The mice received a third week of antibody treatments without additional huNK cell injections. (B) AML engraftment levels in the blood of individual mice over time. (C) Median engraftment levels in the blood over time. (D) EFS curves. CSL362 after chemotherapy significantly improved survival against all other groups. Blue bars mark the time of treatment with AraC/DNR; black bars mark the time of treatment with antibodies and huNK cells. N = 6-8 mice per group.