SUPPLEMENTARY APPENDIX

CD16+NK-92 and anti-CD123 monoclonal antibody prolongs survival in primary human acute myeloid leukemia xenografted mice

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Supplemental Methods and Figures

Supplemental methods (Williams et al.)

Chromium release assay

1x10e6 target cells were resuspended in 200 µl of standard culture medium, and treated with 100 µCi of $Na_2{}^{51}CrO_4$ for 2 hours, and then washed x2 in AIM-V serum free medium prior to treatment with NK-92. 10 000 radio-labelled targets were added to individual wells of a 96 well U bottom plate. NK-92 was added at various concentrations to yield 25:1, 10:1, 5:1 and 1:1 effector:target (E:T) ratios and plates were centrifuged at 500 rpm and incubated at 37°C 5% CO_2 x 24 hrs. Plates were incubated for 4 hr at 37°C in 5% CO2 and centrifuged at 400 *g* for 5 min, and 100 µl of supernatant were collected from each well and transferred into collection tubes. The plates were then centrifuged, supernatants collected and assayed in a gamma counter. The amount of ^{51}Cr present in supernatants was determined using a gamma counter. Percent lysis was calculated using the formula:% lysis = E-S/M-S x100% where E is the ^{51}Cr-release from an experimental sample, S is the spontaneous release in the presence of target cell growth medium and M is the maximum release upon cell lysis with Triton X 100 10%. Data are presented as the mean percent lysis of triplicate samples (+/-SD) from a representative experiment repeated 3 times.

ADCC chromium release assay and antibodies

Target cells were labelled with 100 µCi of $Na_2{}^{51}CrO_4$ for 2 hours with simultaneous incubation with +/- 10 µg/ml of mAb (anti-class I HLA A, B, C or 7G3 or isotype control) prior to treatment with CD16+NK-92 in 96 well plates in a standard chromium release assay as previously described. Antibodies purchased from Biolegend were (clone; product#): Anti-HLA A, B, C

(W6/32; 311412) and isotype controls MG1-45 (MG1-45; 401404) and MG2a-52 (MG2a-53, 401502). 7G3 was purchased from BD biosciences (7G3; 554527). Additional spontaneous and maximal release controls were conducted with and without antibody pretreatment. Plates were centrifuged at 400 *g* and 100 µl of supernatant were collected and assayed in a gamma counter.

Methylcellulose cytotoxicity assay: Effectors and targets were incubated together in 96 well U bottom plates for four hours and spun at 500 rpm (high density conditions) and then infused into methylcellulose (low density conditions) and evaluated at two weeks for cell lines and four weeks for primary AML samples. Controls included tumour alone and tumour and effector incubated in separate wells for four hours and co-infused into methylcellulose (low density control) to determine the impact of effectors on targets in methycellulose under low density conditions (Online supplementary Figure S1). Five primary AML samples (10 000 cells/plate) were incubated with or without NK-92 at 25:1 E:T ratio for four hours in 96 well U bottom plates comparable to a chromium release assay setup. Subsequently cells were transferred into methylcellulose and 10 000 whole AML blasts from each treatment group were transferred to each of three 35 mm plates. Methylcellulose plates were examined for colonies at two weeks and enumerated (colony definition >=50 cells) and number of colonies on each of the three plates per group were averaged and presented +/- SD.

Percent clonogenic inhibition was calculated according to equation (1):

(1) $\%\ 1^0$ Clonogenic Inhibition $= \dfrac{[(\#Colonies_{LDC}) - (\#Colonies_{Treatment})]}{[\#Colonies_{LDC}]} \times 100\%$

$\#Colonies_{LDC}$=the number of colonies in the low density control.

Primary AML xenograft model

We developed a primary AML xenograft model utilizing an AML sample from a single patient with aggressive engraftment potential in NSG mice. The AML sample was M4 with cytogenetic abnormalities (45XY inv3 -7), and an immunophenotype positive for CD4, CD7, CD11, CD13, CD15, CD34, HLA-DR obtained at diagnosis (080719) or first relapse (080315). In addition, the the sample contained a small fraction of CD34+CD38- cells that were predominantly CD123+.

NSG mice were irradiated with 225 or 325 cGy of irradiation followed by immediate injection with 3 $x10^6$ fresh frozen primary AML cells or *in vivo* passaged primary AML derived from the spleens of mice with end stage leukemia. This led to a symptomatic leukemia by week 6. Leukemic progression led to significant weight loss, bone marrow infiltration with human leukemia, anemia and splenomegaly. Further, potency of serially transplanted BM or SPL cells from NSG with end stage leukemia was maintained and could lead to leukemia in mice given secondary, tertiary or quaternary transplants with the same kinetics of freshly thawed primary AML samples from the leukemia bank (Online supplementary Figure S). Spleen derived AML was as potent as that derived from BM with no reduced engraftment (data not shown). We utilized this model to assess the *in vivo* efficacy of NK-92 as described below. NK-92 cells were infused via tail vein or intraperitoneally on a dose and schedule that varied according to experiment. Cages of mice from of similar age were randomized for all animal experiments and most cohorts consisted of 5 mice per treatment group or control. Mice were monitored for leukemia and sacrificed at humane endpoints. Autopsies were performed on select mice from each cohort, including flow cytometry of bone marrow (BM) and splenocytes to determine leukemic burden.

Secondary transplantation model

To test more rigorously if NK-92 had any impact on leukemic stem cells we establish a secondary transplant model. We utilized primary AML sample (080315) which had potential to passage up beyond secondary transplantation as described above. Primary AML cells ($3x10^6$) were injected by tail vein into two cohorts of four mice and treated with or without iNK-92 from day 2 ($15x10^6$ iNK-92 cells) twice weekly for 5 doses. At six weeks, mice in both cohorts were sacrificed and $1x10^6$ bone marrow cells from each of the 4 primary recipients (donor mice) in control or treatment groups were transplanted into four secondary recipient NSG mice. BM was transferred in a 1:1 fashion from the primary transplant group mice to the secondary transplant group mice. At 6 weeks after the secondary transplantation mice were sacrificed and bone marrow assessed by flow cytometry to measure engraftment of leukemic cells (CD45+), as well as the leukemic stem cell fraction (CD34+CD38-CD123+).

Cell Sorting

Primary AML samples were stained with anti-CD34 PE and anti-CD38 APC (BD Biosciences) for cell sorting using a FacsAria cell sorter. 10 million fresh primary AML blasts were thawed, washed and resuspended in alpha-MEM +20% FBS +10% 5637 conditioned medium. Viability was assessed using trypan blue exclusion test. Samples were then washed in 10 ml of FACS buffer (PBS, 1% FBS, 2 mM EDTA), supernatant discarded and resuspended in 2 ml FACS buffer (~$5x10^6$ cells/ml). Cells were then filtered using 5ml polystyrene round tube with cell-strainer caps (BD Falcon). Samples were split samples into five groups to use in setting gates: not sorted control, untreated control (ie no antibody treatment, but run through sorter), CD34 PE

control, CD38 APC control, CD34 PE + CD38 APC control. Following antibody addition, samples were incubated at room temperature for 20-30 minutes in the dark, washed with 10 ml of FACS buffer and resuspended in 1-2 ml of FACS buffer to yield 5 million cells/ml. Following establishment of the CD34+CD38+ and CD34+CD38- gates, remaining primary AML cells with double staining were sorted into these two fractions for later use in a chromium release assay as outlined previously. Purity checks were conducted to confirm the efficacy of the sorting procedure and viability of cells were again determined with trypan blue exclusion assay prior prior to use in chromium release assay.

Supplemental figures (Williams et al.)

Figure S1

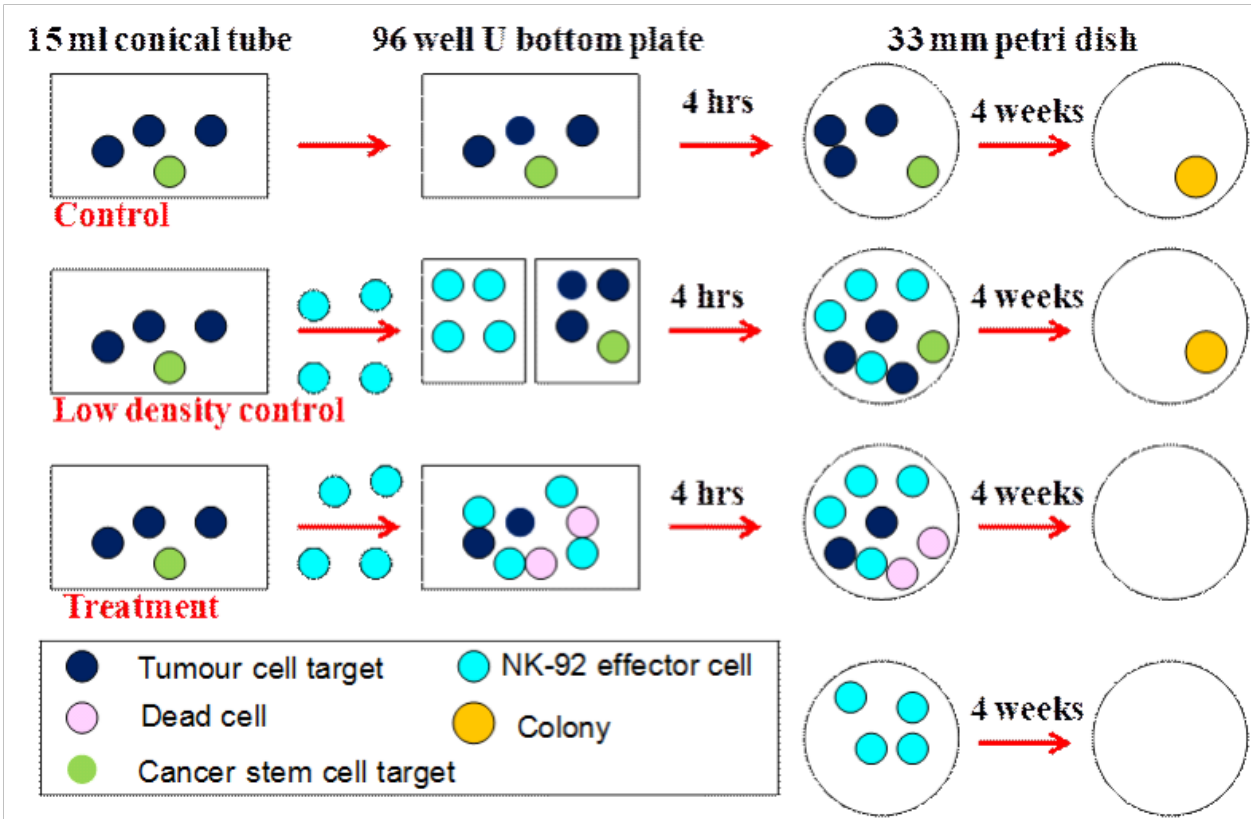

Figure S1: **Schematic of the methylcellulose cytotoxicity assay.**

Figure S2

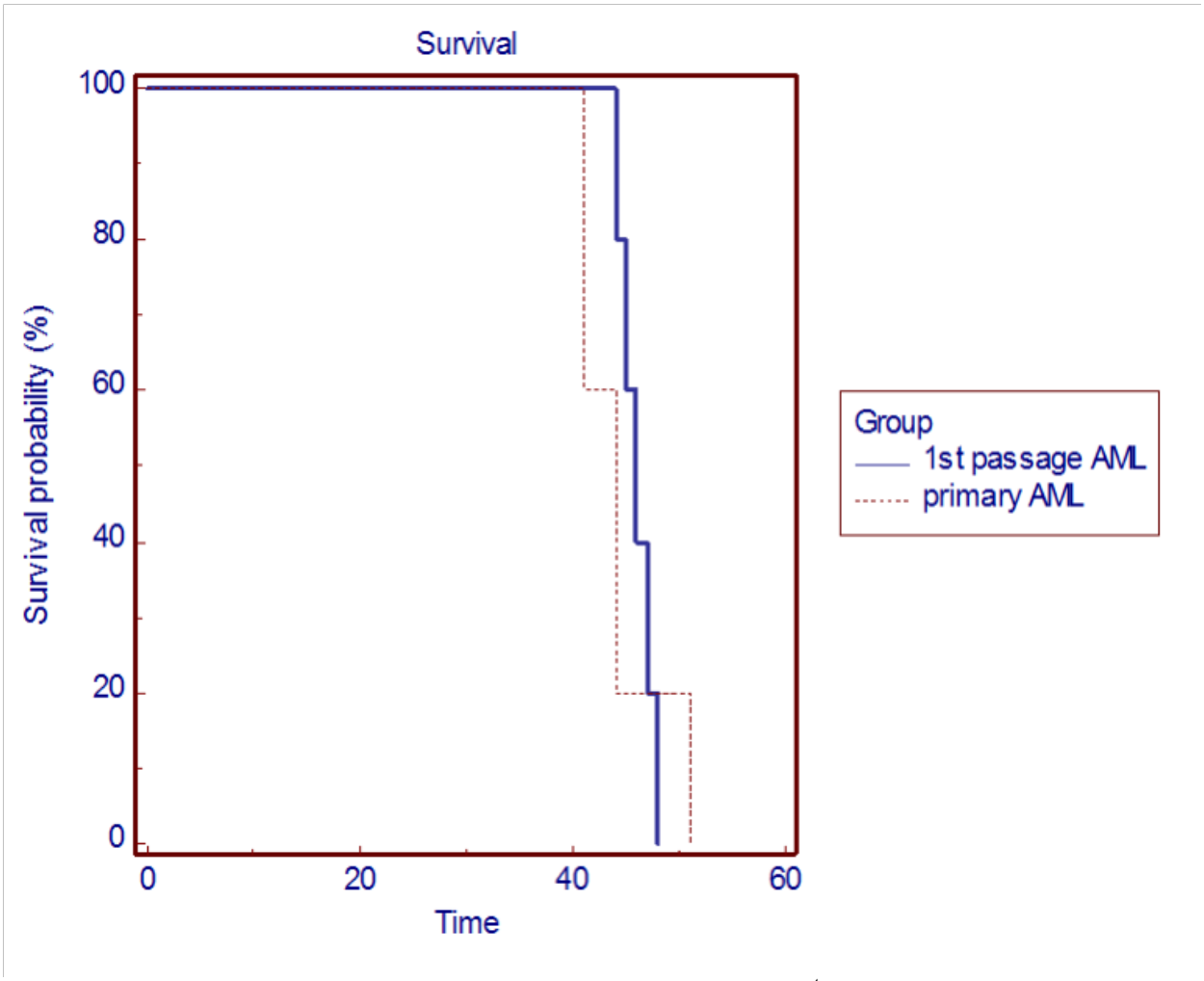

Figure S2: **Survival of NSG mice with primary AML versus 1st passage AML derived from BM**

NSG mice were infused with 3x10e6 freshly thawed primary AML (080791) or spleen derived first passage AML (n=5 per group). Mice were monitored until humane endpoint and sacrificed. Survival was analyzed using Kaplan Meier curves and log rank test (p= NS).

Figure S3

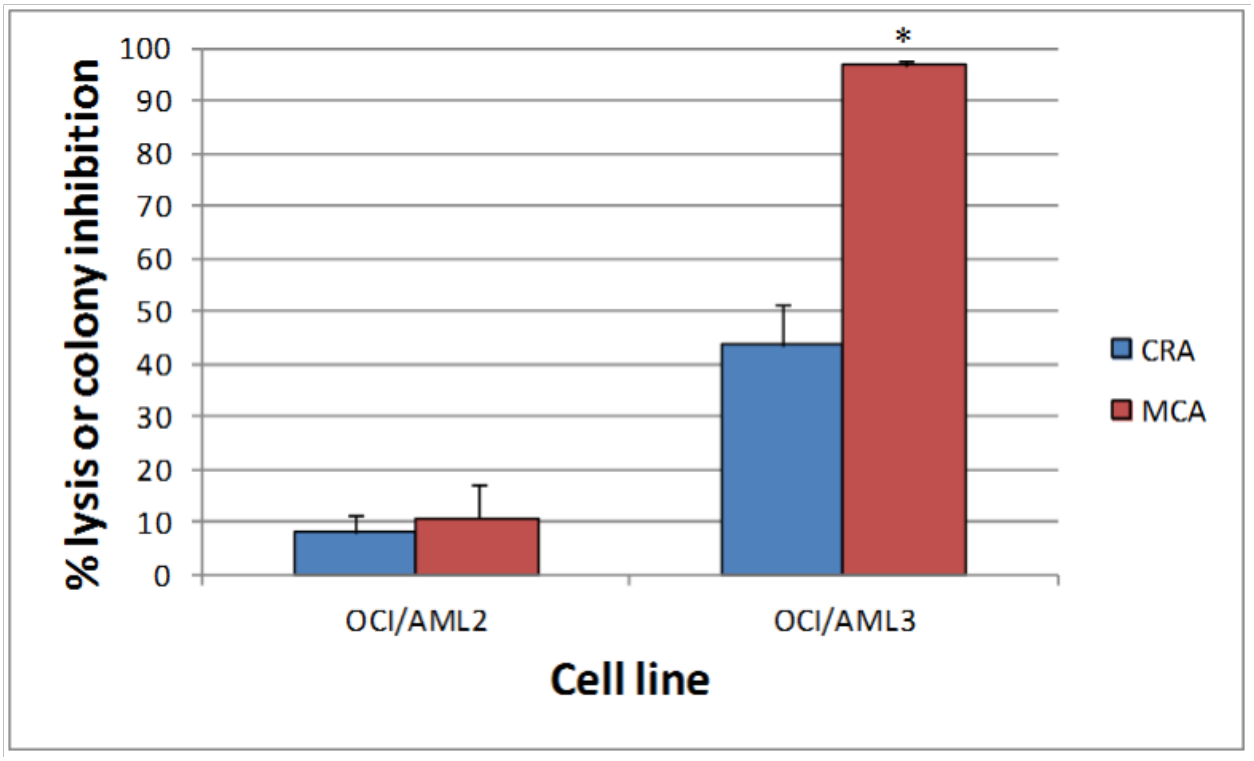

Figure S3: **Clonogenic cytotoxicity assay of NK-92 against OCI/AML2 and OCI/AML3**

NK-92 cytotoxicity was determined against cell lines OCI/AML2 and OCI/AML3 using a methylcellulose cytotoxicity assay (MCA) and chromium release assay (CRA) both with four-hour incubations. For the CRA, OCI/AML2 and OCI/AML3 were labelled with 100 µCi of $Na_2{}^{51}CrO_4$ for 2 hours prior to treatment with NK-92 at a 25:1 E:T in 96 well U bottom plates. For the MCA, targets cells, target cell + effector cells incubated separately and target cells and effector cells incubated together were infused into methylcellulose. Colonies were quantified by inverted microscopy following 10 days at 37°C incubation. CRA is presented as % lysis and MCA results are presented as % colony inhibition. Data are presented as the mean of triplicate samples (+/-SD) from a representative experiment repeated 3 times. *= $p<0.05$.

Figure S4

A **B** **C**

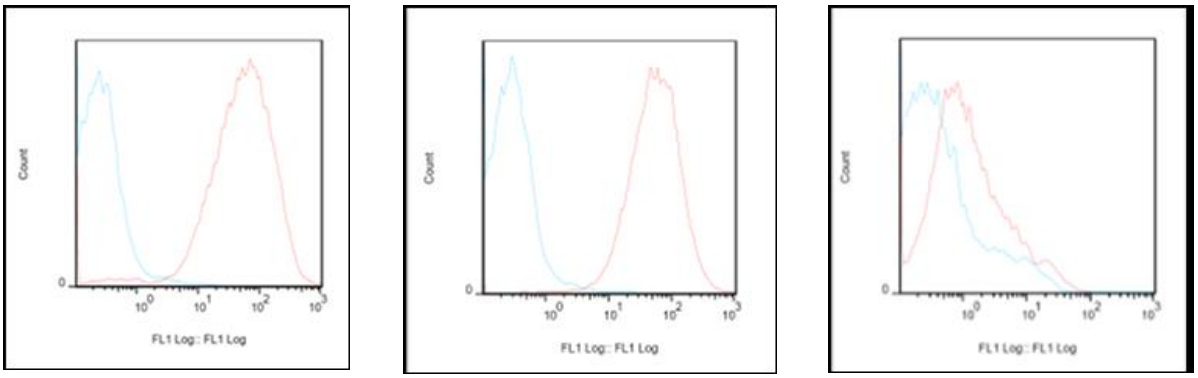

Figure S4: Autopsies were performed on select mice from each cohort from the experiment depicted in Figure 4 with AML xenografted mice treated with NK-92. Flow cytometry of BM was done to determine leukemic engraftment. Unstained (blue) and anti-Class I FITC stained (red) specimens are presented from one mouse with symptomatic leukemia (A), a mouse with AML in the treatment group that developed symptomatic leukemia (B) and a healthy long-term survivor (C).

Figure S5

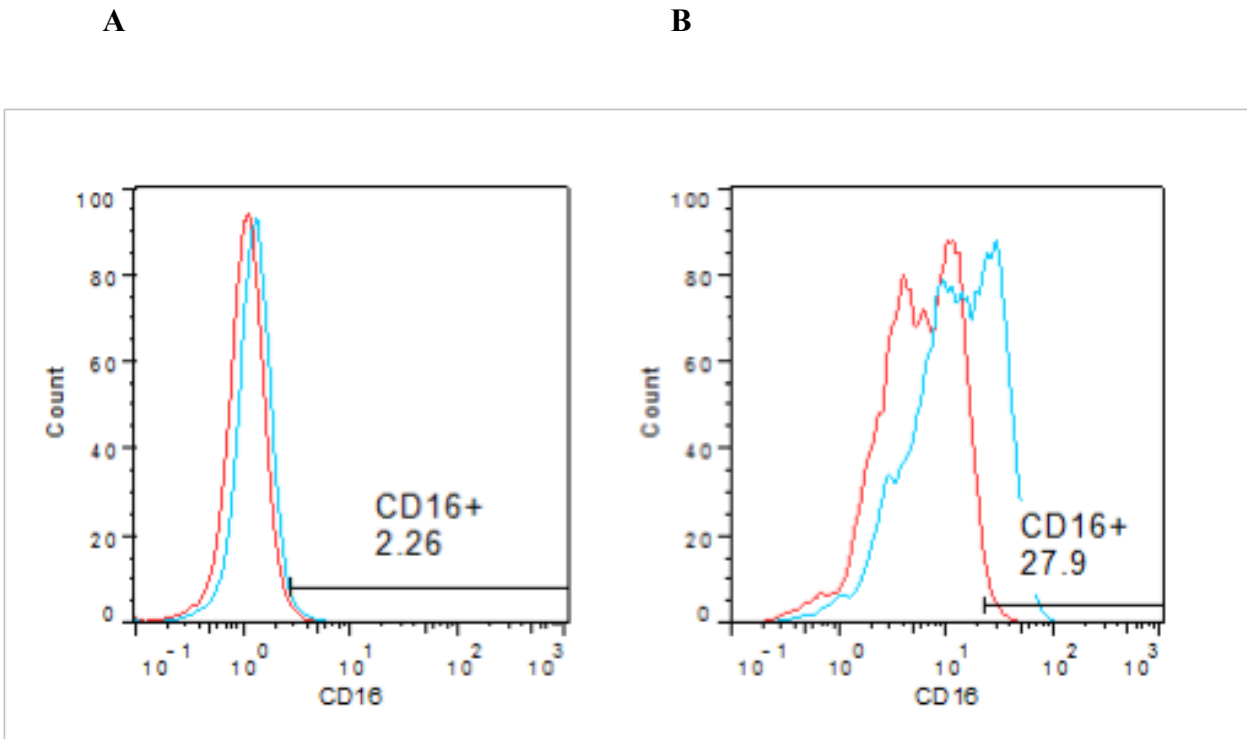

Figure S5: **Immunophenotyping of CD16+NK-92, NK-92**

Immunophenotyping of NK-92 (A) and CD16+NK-92 (B) after staining with CD16 PE. Samples were run on an FC500 flow cytometer. Unstained (red) and stained (blue) populations are presented in the histogram. Gates were set to exclude 99% of unstained events for each cell line to define positive and negative populations.

Figure S6

A

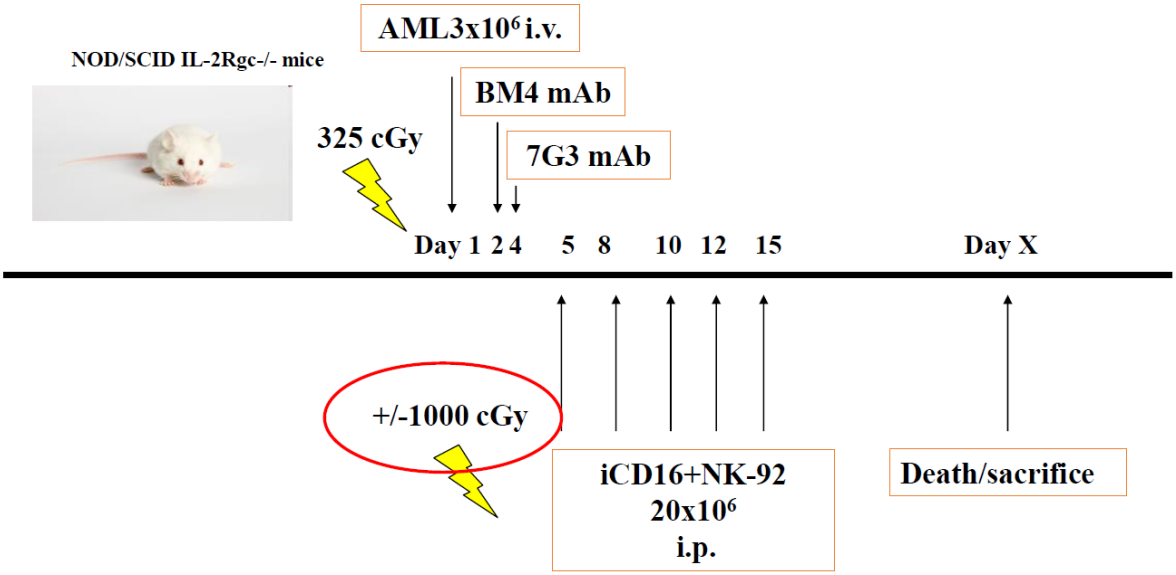
AML3x10⁶ i.v.
NOD/SCID IL-2Rgc-/- mice
BM4 mAb
325 cGy
7G3 mAb
Day 1 2 4 5 8 10 12 15
Day X
+/-1000 cGy
iCD16+NK-92
20x10⁶
i.p.
Death/sacrifice

Figure S6

B

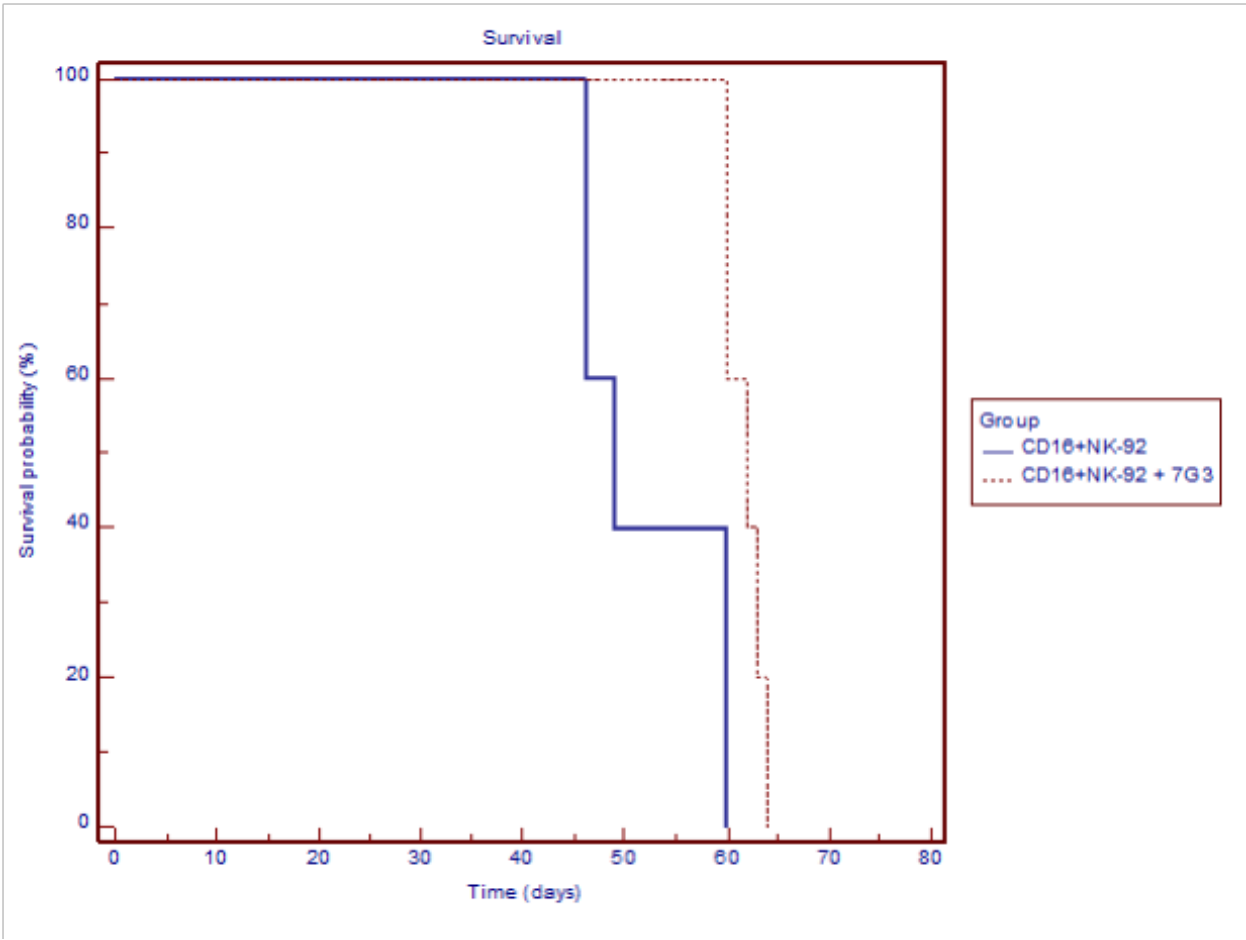

Figure S6: iCD16+NK-92 +/- a single anti-CD123 mAb dose for primary AML xenografted NSG mice

NSG mice (n=5 per group) were inoculated with $3x10^6$ passage human AML spleen derived cells (day 0) and treated with iCD16+NK-92 x 5 doses +/- 8 µg 7G3 starting on day 3 (A). Survival was determined using Kaplan Meier survival analysis (B) and differences evaluated with a log rank test (p=0.0173).