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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ABSTRACT

The prognosis of older patients with acute myelogenous leukemia is generally poor. The interleukin-3 receptor α-chain (CD123) is highly expressed on the surface of acute leukemia cells compared with normal hematopoietic stem cells. CSL362 is a fully humanized, CD123-neutralizing monoclonal antibody containing a modified Fc structure, which enhances human natural killer cell antibody-dependent cell-mediated cytotoxicity. Six continuous acute myelogenous leukemia xenografts established from patient explants and characterized by cell and molecular criteria, produced progressively lethal disease 42-202 days after transplantation. CSL362 alone reduced engraftment of one of four and three of four acute myelogenous leukemia xenografts in the bone marrow and peripheral organs, respectively. A cytarabine and daunorubicin regimen was optimized using this model to identify potentially synergistic interactions with CSL362. Cytarabine/daunorubicin improved the survival of mice engrafted with four of four acute myelogenous leukemia xenografts by 31-41 days. Moreover, CSL362 extended the survival of cytarabine/daunorubicin-treated mice for two of two acute myelogenous leukemia xenografts, while augmentation of natural killer cell-deficient NSG mice with adoptively transferred human natural killer cells improved survival against a single xenograft. Interestingly, this enhanced CSL362 efficacy was lost in the absence of chemotherapy. This study shows that acute myelogenous leukemia xenografts provide a platform for the evaluation of new therapeutics, simulating complex *in vivo* interactions, and that the *in vivo* efficacy of CSL362 supports continued clinical development of this drug.

Introduction

Contemporary protocols for the treatment of acute myelogenous leukemia (AML) are able to cure 10-60% of patients, with those in older age groups having a poorer prognsois.[1,2] Standard therapy includes cytarabine (AraC) for 7 days and daunorubicin (DNR) for 3 days (the "7 + 3" regimen). The addition of novel drugs to the 7 + 3 regimen, such as fludarabine,[3] cyclosporine[4,5] and zosuquidar,[6] has produced varied clinical benefit, and new therapies are required. Antibody targeting therapy is a promising approach with high specificity and low toxicity.

The interleukin-3 receptor α-chain (CD123) is highly expressed on AML stem cells but not hematopoietic stem cells,[7-11] providing a target for antibody-based therapy. 7G3 is a mouse neutralizing monoclonal antibody targeting human CD123,[12] which demonstrated activity against AML-leukemic stem cells, reduced AML burden and improved survival of engrafted mice.[8] The human chimeric version of this antibody, CSL360, was tested in AML patients in a phase I clinical trial in which it demonstrated specificity and safety but did not have clear anti-leukemic activity.[13] While CD123 remains a therapeutic target for AML, its neutralization was not suffi-

cient for clinical benefit. CSL362 is based on the CSL360 antibody but is fully humanized, affinity matured, and contains specific Fc-domain point modifications to enhance binding affinity with Fcγ receptors (FcγR)[14] and augment anti-leukemic activity via antibody-dependent cellular cytotoxicity (ADCC) against leukemic blasts and leukemic stem cells.[15]

Human natural killer (NK) cells express CD16 (FcγRIIIa), which binds the Fc portion of antibodies.[16] Once ligated, CD16 associates with FcεRIγ or CD3ζ resulting in transduction of an activation signal.[17] NK cell responses to target cells are governed by the balance of cell surface activating and inhibitory receptors. There is evidence to indicate that NK cells play an important role in leukemia eradication in patients. In cases of haploidentical stem cell transplants that had mismatched inhibitory ligands to the NK cell receptors, improved survival of patients was observed.[18-20] Adoptive transfer of NK cells in mice also delayed the progression of cancer cell lines *in vivo*.[21-23]

In this study, six continuous AML xenografts with defined cytogenetics and mutations, which produced clinically relevant progressive lethal disease in immune-deficient mice, were established from AML apheresis samples. A regimen of AraC and DNR modeling the clinical 7 + 3 schedule was opti-

mized, which improved the survival of AML engrafted mice, providing a platform for preclinical examination of CSL362. Since the compromised NK cell function of immune-deficient mice may underestimate the efficacy of CSL362, human buffy coat-derived NK cells were expanded *ex vivo* for adoptive transfer into AML xenografted mice. CSL362 demonstrated additional efficacy against AML xenografts in combination with chemotherapy and adoptively transferred human NK (huNK) cells, supporting its further development in the clinic.

Methods

Samples from patients with acute myelogenous leukemia, xenograft cells, and cell lines

Apheresis samples were collected from AML patients after informed consent and studies were approved by the Royal Adelaide Hospital Human Ethics Committee, Melbourne Health Human Research Ethics Committee and South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee in accordance with the Declaration of Helsinki. The demographics, cytogenetics and clinical data of the AML patients are described in Table 1. The Epstein-Barr virus-transformed B lymphoblastoid (SMI-LCL)[24] and K562-mIL15-41BBL[22] cell lines were cultured as previously described.

Xenotransplantation and in vivo drug treatments

All mice were maintained under protocols approved by the Animal Care and Ethics Committee of the University of New South Wales. Transplantation of human cells into female NOD/SCID (NOD.CB17-*Prkdc^scid^/J*) or NSG (NOD.Cg-*Prkdc^scid^Il2rg^tm1Wjl^/SzJ*) mice was performed as previously described, and patients' samples that produced aggressive and highly infiltrative disease were prioritized for development into continuous xenografts.[8] Briefly, mice were exposed to sub-lethal irradiation (250 cGy, delivered by X-RAD 320, Precision X-Ray, CT, USA) 1 day before intravenous (IV) injection of $2–10 \times 10^6$ human AML cells via a heat-dilated tail vein. Engraftment levels were quantified by flow cytometry and expressed as the percentage of human $CD45^+$ ($hCD45^+$) cells to total $hCD45^+$ and mouse $CD45^+$ ($mCD45^+$) cells in the tissue sample.[25] AML cells were distinguished from huNK cells by dual staining for CD45 and CD33. Hematology was analyzed on a Coulter® AcT·diff™ Analyzer (Beckman Coulter, CA, USA). Blood was obtained either by cardiac puncture or tail vein bleeds. *In vivo* drug treatment protocols are described in the *Online Supplement*.

Mutation analyses of acute myelogenous leukemia xenografts

The mutant:wild-type allelic burden of *FLT*-internal tandem duplication (*FLT3*-ITD) and nucelophosmin (*NPM1*) in genomic DNA was analyzed using modifications of the methods of Murphy *et al*.[26] and Huang *et al*.[27] Polymerase chain reaction products were resolved by capillary electrophoresis (AB 3130 analyzer, Applied Biosystems). For *FLT3*-ITD, mutant to wild-type peak area ratios ≥0.05 were considered positive and are expressed as percentages in Table 1. The mixed lineage leukemia-partial tandem duplication (*MLL*-PTD) was determined using a reverse transcriptase real-time quantitative polymerase chain reaction method, in which the *MLL*-PTD was quantified as the ratio of the *MLL*-PTD concentration (fg/μL) to *ABL1* gene concentration (ag/μL) x 10^{-3}.

A multiplexed matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) genotyping approach (Sequenom MassARRAY Compact System) was used to detect mutations affecting *c-KIT* D816, *DMNT3A* R882, *FLT3*-TKD, *IDH1* R132, *IDH2* R140/R172, *JAK2* V617, *KRAS* G12/13, *MPL* W515, *NPM1* W288, and *NRAS* G12/13 and Q61 using custom designed sequences (available on request). Polymerase chain reaction products were prepared using Sequenom iPLEX Pro following published methods, spotted onto a SpectroChip II matrix and resolved using mass spectrometry.[28]

Isolation and expansion of human natural killer cells and the antibody-dependent cellular cytotoxicity assay

NK cells were isolated from human buffy coats (Australia Red Cross Blood Service, Sydney, Australia), expanded and activated as previously described,[24, 29] with modifications as described in the *Online Supplement*.

Additional details of the methods are provided in the *Online Supplement*.

Results

Development and characterization of a panel of continuous acute myelogenous leukemia xenografts

A panel of six continuous xenografts was established in immune-deficient mice from AML patients' explants with heterogeneous clinical characteristics, cytogenetics and mutations, although four of the six harbored alterations in the *FLT3* gene (Table 1). AML apheresis samples known to produce highly infiltrative disease were inoculated into NSG or NOD/SCID mice and expanded for up to six serial passages by transferring human leukemia cells harvested from the spleens, bone marrow, and livers of engrafted mice into secondary recipient mice (Figure 1A, *Online Supplementary Figure S1A*). A sufficient number of human leukemia cells could not be routinely harvested from mice inoculated with AML-1 in order to establish a continuous xenograft.

Mutations affecting *c-KIT*, *DNMT3A*, *FLT3*-ITD, *FLT3-TKD*, *IDH1*, *IDH2*, *JAK2*, *c-MPL*, *NPM1*, *NRAS*, *KRAS*, and *MLL*-PTD were assessed in the xenografts and diagnostic primary samples (Table 1). AML-4 appeared to be derived from an ancestral clone, with the same *FLT3*-TKD and *IDH2* mutations present in both xenograft and primary samples, although the *FLT3* mutant:wild-type ratio was notably higher in the xenograft. Similarly, AML-5 also appeared to be derived from an ancestral clone expressing the *WT1* synonymous single nucleotide polymorphism rs16754, although a low level *FLT3*-ITD subclone detected in the primary sample was lost in the xenograft. The AML-9 primary sample contained at least four separate *FLT3*-ITD subclones, with a minor *FLT3*-ITD subclone selected in the xenograft along with loss of *WT1* rs16754. Similarly, the AML-16 primary and xenograft expressed identical mutations in *IDH2*, *NPM1*, and *WT1*, although the primary sample contained two *FLT3*-ITD subclones, the larger of which was selected in the xenograft. The AML-17 primary sample consisted of dominant (63 bp) and minor (45 bp) *FLT3*-ITD subclones, although the xenograft expressed a unique 48 bp *FLT3*-ITD subclone that could not be detected in the primary sample even after two rounds of polymerase chain reaction amplification. The AML-17 xenograft also harbored an *MLL*-PTD, which was detected at an extremely low level (0.08) in the primary sample. Lastly, the AML-18 primary sample con-

Table 1. Patients' characteristics, cytogenetics, and mutations of AML xenografts.

Xenograft designation	Sample	Age(yr)/ Sex	AML subtype[a]	Cytogenetics	WCC[b] (x10⁹/L)	*FT3* ITD Pos/Neg	*FLT3* ITD Allelic load	*FLT3* ITD size in bp (allelic (%) load)	Summary of mutation analysis (mut:wt ratio)[c]
AML-1	Primary	69/F	M1	Normal	135	Pos	37.3	54 (2.8%); 60 (34.5%)	*IDH2* c.419G>A p.R140Q (1.36)
AML-4	Primary	76/M	M5a	Trisomy 8	227	Neg	–	–	*FLT3* c.2503G>T p.D835Y (1.05) *IDH2* c.419G>A p.R140Q (1.23)
	Xenograft					Neg	–	–	*FLT3* c.2503G>T p.D835Y (1.76) *IDH2* c.419G>A p.R140Q (1.24)
AML-5	Primary	19/F	M2	Monosomy 7	108	Pos	9.7	57	*WT1*_16754 (0.60)
	Xenograft					Neg	–	–	*WT1*_16754 (0.86)
AML-9	Primary	51/M	M4Eo	Inv(16)	171	Pos	36.0	33 (31.2%); 54 (2.5%); 63 (1.9%); 69 (0.6%)	*WT1*_16754 (0.60)
	Xenograft					Pos	50.4	54	No mutations detected
AML-16	Primary	61/F	M4	Normal	19	Pos	87.2	12 (1.2%); 18 (86%)	*IDH2* c.419G>A p.R140Q (1.25) *NPM1* c.1165G>T (Type A) (0.75) *WT1*_16754 (0.55)
	Xenograft					Pos	100	18	*IDH2* c.419G>A p.R140Q (1.05) *NPM1* c.1165G>T (Type A) (0.78) *WT1*_16754 (0.76)
AML-17	Primary	85/M	M4	Trisomy 8	179	Pos	86.5	63 (dominant) 45 (minor)	*MLL*-PTD (0.08)[d]
	Xenograft					Pos	100	48	*MLL*-PTD (12.7)
AML-18	Primary	43/F	M1	t(9;11), MLL translocation	221	Neg	–	–	*KRAS* c.35G>C p.G12A (1.19)
	Xenograft					Neg	–	–	*KRAS* c.35G>C p.G12A (0.71) WT1_16754, (0.12)

IDH2: *isocitrate dehydrogenase;* ITD: *internal tandem duplication;* KRAS: *Kirsten rat sarcoma;* MLL: *mixed lineage leukemia;* mut, *mutant;* NPM: *nucleophosmin;* PTD: *partial tandem duplication;* WCC: *peripheral blood white cell count;* wt: *wild-type;* NA: *not available.* [a]*French-American-British criteria;* [b]*At diagnosis;* [c]*By Sequenom and capillary electrophoresis;* [d]MLL-PTD *reference range, <3 negative, 3-10 equivocal, >10 positive.*

sisted of a dominant *KRAS*-mutated clone, which was also dominant in the xenograft, but with the appearance of a minor *WT1* rs16754 subclone. These results indicate that the overall mutational spectrum of these AML samples was conserved upon xenografting, along with variability in the selection of subclones depending on the xenograft.

AML xenografts produced progressive disease that was monitored in the peripheral blood, and engraftment rates accelerated in subsequent passages in five of six AML xenografts (Figure 1A, *Online Supplementary Figures S1A and S2* and *Online Supplementary Table S1*). Engraftment of the AML-17 biopsy did not reach the predefined event point after 163 days, although cells harvested from the bone marrow subsequently produced a continuous xenograft.

Cell surface expression of CD33 and CD45 was maintained on the AML xenograft cells (Figure 1B, *Online Supplementary Figure S1B*). Moreover, there were no overt changes in cell surface CD34, CD38 and CD123 expression between the patients' samples and the respective xenografts except for AML-4 and AML-17 xenografts. AML-17 acquired a CD123^{hi} population and AML-4 became CD34^{-}. In patients' biopsies AML-4, -9, -16, and -17, the CD33^{-} population was CD3^{+} (*data not shown*), which disappeared upon secondary transplantation.

NSG mice are more receptive hosts for human xenografts than NOD/SCID mice[30] and this was observed with AML-9 and AML-18 xenografts (*Online Supplementary Figure S3* and *Online Supplementary Table S1*). Furthermore, for AML-9 and AML-17, only three of five and three of six NOD/SCID mice, respectively, developed a human AML disease due to the early manifestation of spontaneous murine thymic lymphoma inherent in this mouse strain.[31] In contrast, all NSG mice inoculated with AML-9, -17 and -18 developed AML.

We also tested the requirement for sub-lethal irradiation of NOD/SCID mice for engraftment using AML-5. While four of four irradiated mice reached an event at a median of 63 days (Figure 2A and *Online Supplementary Table S1*), AML cells were not detected in the peripheral blood of two of three non-irradiated mice at the termination of the experiment, and only one of the mice had trace levels of leukemia in the bone marrow, spleen and liver (*data not shown*). Surprisingly, when we repeated the study with NSG mice, irradiation significantly decreased the rate of engraftment of three of three different AML xenografts tested (Figure 2B and *Online Supplementary Table S1*), albeit in small numbers of mice per AML xenograft.

Mice engrafted with continuous AML xenografts developed "clinical" symptoms and signs associated with leukemia, such as lethargy, loss of body mass, breathing difficulties, and paleness, which if allowed to progress
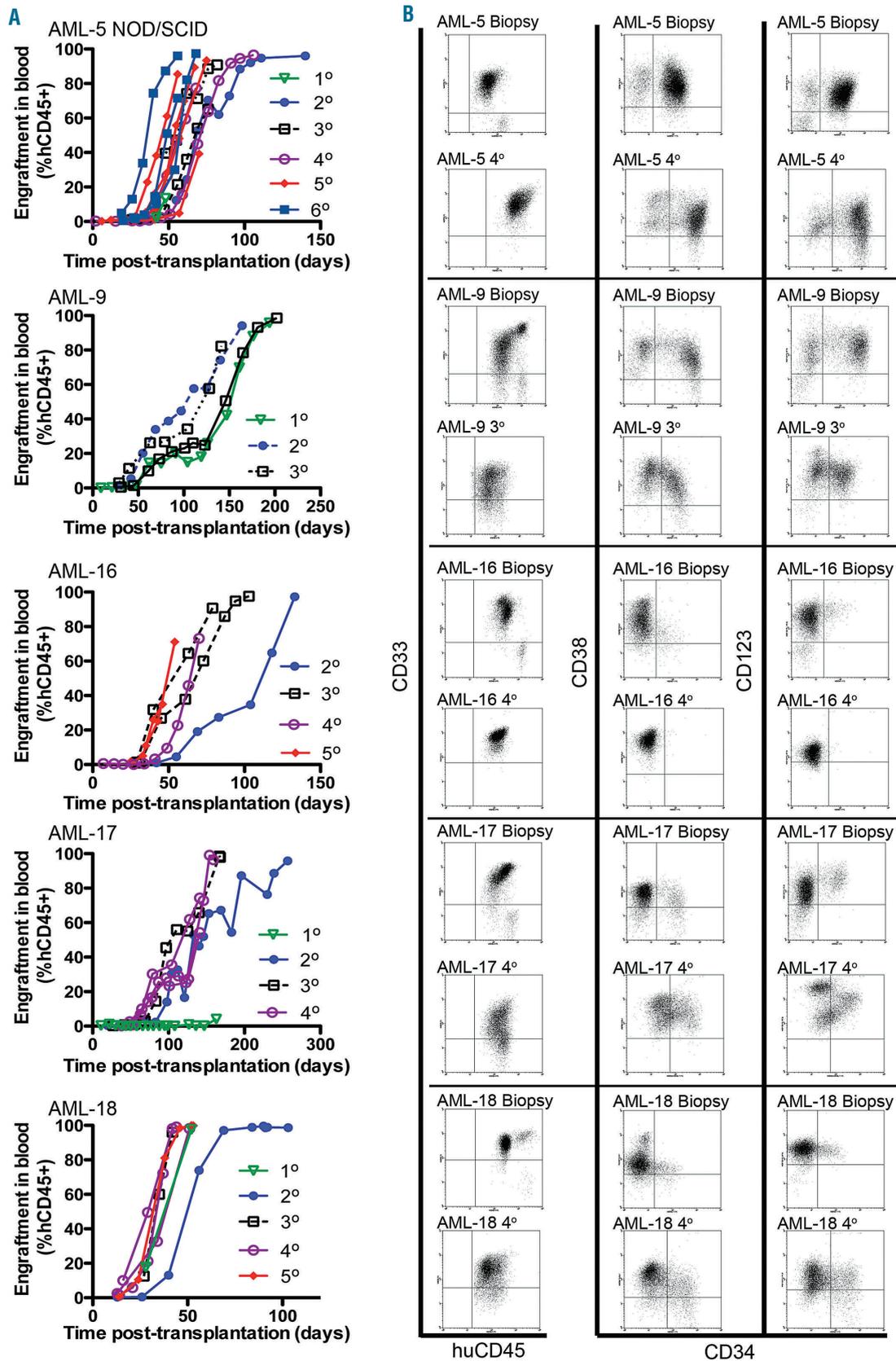
Figure 1. AML xenograft engraftment in blood and immunophenotypes in immunodeficient mice. (A) The engraftment of different passages of AML xenografts in the blood over time post-transplantation. NSG mice were used for all AML xenografts except AML-5 for which NOD/SCID mice were used. Each line represents the median engraftment level for an individual experiment with 2-16 mice per experiment. (B) Representative scatter plots of immunophenotypes of the original patients' samples and respective xenografts 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.

would have resulted in death. The red blood cell count, hematocrit, hemoglobin concentration, and platelet count all correlated inversely ($P<0.0001$) with the %hCD45+ cells in the blood of AML-5 and AML-16 engrafted mice (Figure 2C). The white cell count initially decreased due to the disruption of normal hematopoiesis, and subsequently increased as the leukemia blast cells become numerically dominant. The disruption of hematopoiesis by AML engraftment was further highlighted by a decrease in platelet count before leukemia cells appeared in the blood (Figure 2C). At autopsy, splenomegaly, hepatomegaly, and occasional thymic enlargement were apparent, dependent on the specific AML xenograft. For example, AML-9 consistently formed tumors around the bladder, ovaries and small intestinal regions with thickening of stomach walls, while AML-16 produced exceedingly large spleens (*Online Supplementary Figure S4*). The surface expression of markers associated with hematopoietic cell "stemness" (CD34, CD38 and CD123) was assessed on AML xenograft cells harvested from various organs. Remarkably, within a specific engrafted mouse, there were substantial differences

in the immunophenotype of AML cells recovered from different organs. For example, the proportion of CD34+CD38- AML-5 cells was significantly higher in the liver, blood and spleen than in the bone marrow, while AML-17 CD123 expression was significantly higher in the bone marrow than in the liver, blood and spleen (*Online Supplementary Figure S5*). Taken together these results indicate that the %hCD45+ cells in the blood provides a surrogate indicator of overall disease burden in the mice.

Optimization of an induction-type, cytarabine/daunorubicin chemotherapy regimen in mice xenografted with human acute myelogenous leukemia

In order to identify possible synergistic or antagonistic interactions of novel treatments in combination with established therapy for AML, we optimized an induction-type "4+1" regimen for the xenograft models. The rationale behind the development of this regimen was to induce temporary remissions in leukemia progression (30-40 days) that could then be used as a platform to combine with novel therapies. Engraftment of AML-1 in the murine
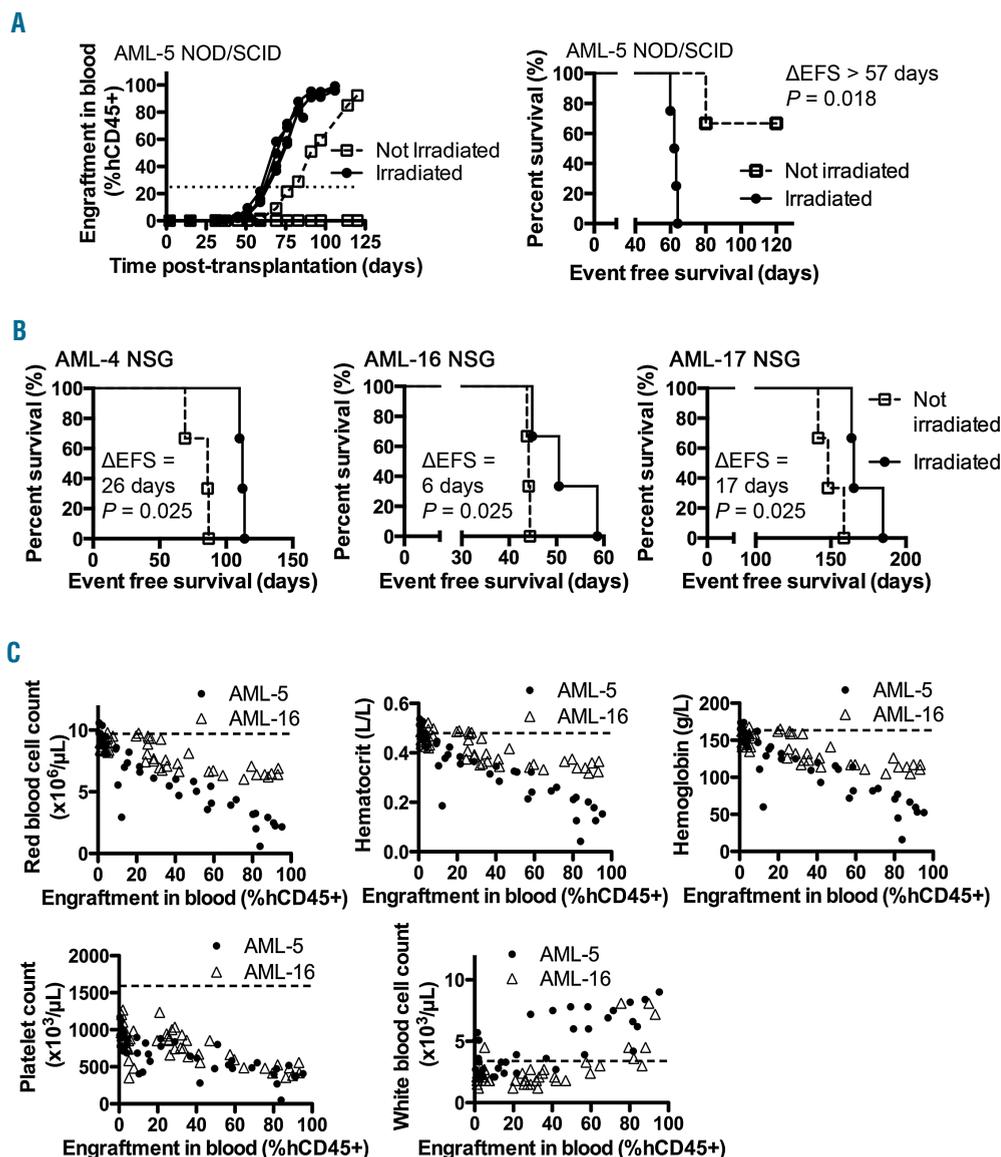
Figure 2. Effects of sub-lethal whole body irradiation on AML engraftment in NOD/SCID and NSG mice, and effects of engraftment on hematology. (A) Engraftment levels of AML-5 in the blood over time of irradiated and non-irradiated NOD/SCID mice and respective event-free survival curves. Each line represents the engraftment level of an individual mouse (B). Event-free survival curves of AML-4, AML-16, and AML-17 transplanted NSG mice with or without irradiation before transplantation. (C) Changes in hematologic parameters with AML-5 and AML-16 xenograft progression. Each individual point represents data from an independent mouse blood sample obtained at different time points; four to eight mice per experiment, results for AML-5 were from two independent experiments. The dashed lines represent respective hematologic parameters obtained from non-irradiated, non-engrafted control mice from four independent experiments with three to five mice per experiment.

bone marrow was significantly reduced following treatment with this AraC (12.5-50 mg/kg/dose) and DNR (0.32-1.25 mg/kg/dose) regimen (Figure 3A). This regimen was further tested on the panel of continuous AML xenografts at a dose of 25 mg/kg AraC and 0.63 mg/kg DNR for 2-3 weeks, which caused substantial decreases in absolute hCD45+ and mCD45+ cell counts in the blood. After the termination of treatments, mCD45+ cell counts rapidly recovered relative to human blast counts, producing a remission-like state. However, the AML blasts eventually overtook the murine hematopoietic recovery producing the final signs and morbidity of the disease (*Online*

Supplementary Figure S6). This dynamic interplay between normal hematopoietic cells and leukemic blasts can be represented as %hCD45+ engraftment, and this regimen of AraC/DNR resulted in significant delays in leukemia progression, and prolonged event-free survival for four of four xenografts (Figure 3B).

Efficacy of cytarabine/daunorubicin or CSL362 as monotherapy against acute myelogenous leukemia xenografts *in vivo*

The effects of AraC/DNR on AML engraftment in bone marrow, spleen, liver and blood were measured in AML-5
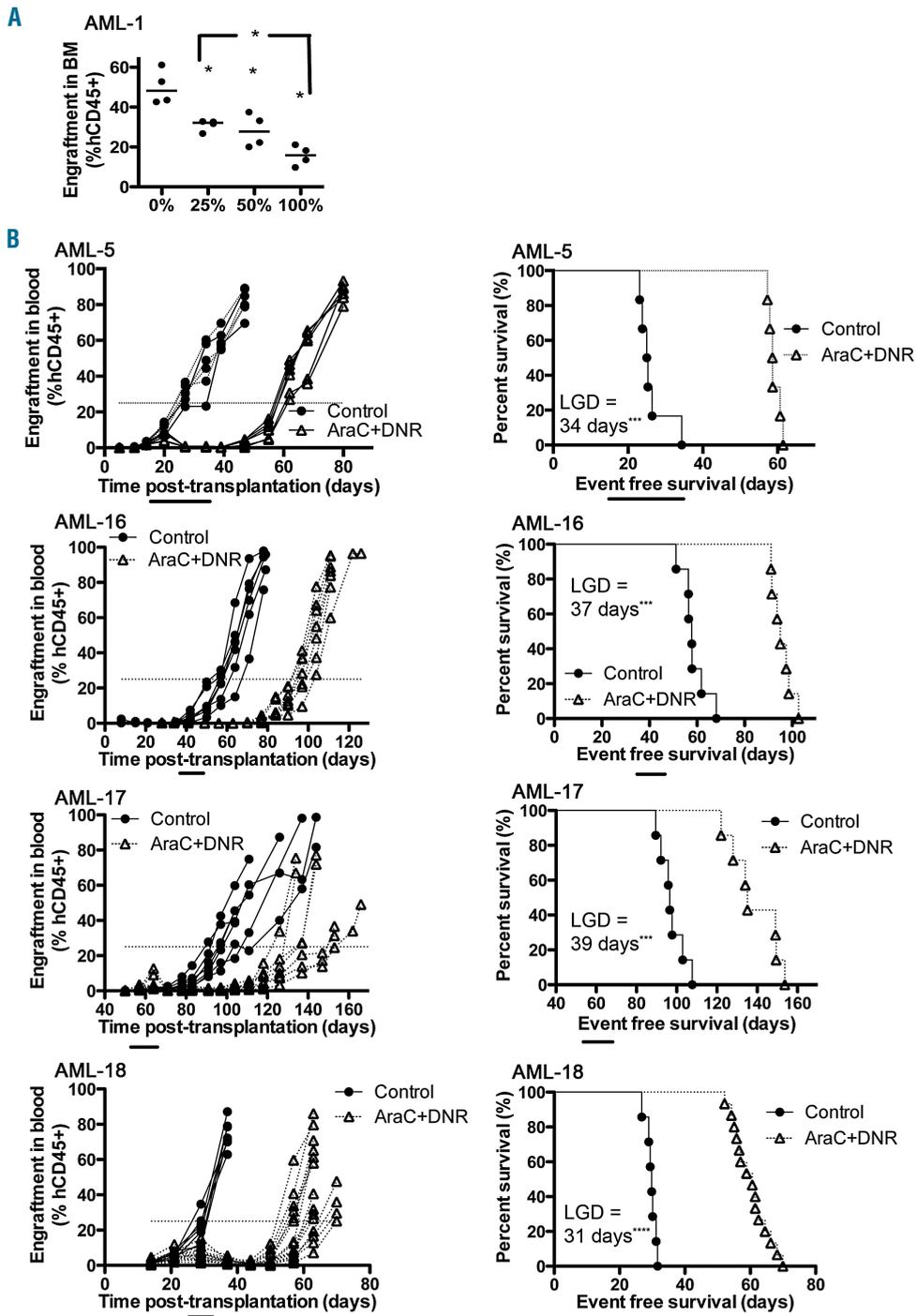
Figure 3. AML xenograft responses to a "4+1" regimen of AraC and DNR in immunodeficient mice. (A) Engraftment levels of AML-1 xenograft cells in the bone marrow (BM) of NOD/SCID mice in response to AraC/DNR dose combinations. 100% represents 50 mg/kg of AraC and 1.25 mg/kg of DNR; 50% (25 mg/kg AraC, 0.63 mg/kg DNR) and 25% (12.5 mg/kg AraC, 0.32 mg/kg DNR) doses were also tested. The mice received three different doses of the AraC/DNR regimen for 2 weeks over days 14-25 after transplantation, and the mice were sacrificed on day 28. Each point represents data from a single mouse; four mice per group. Horizontal bars represent median values. (B) Engraftment levels in blood and respective event-free survival curves for four different AML xenografts treated for 2-3 weeks with AraC (25 mg/kg) and DNR (0.63 mg/kg) in NOD/SCID (AML-17), and NSG (AML-5, 16, and 18) mice. LGD = leukemia growth delay, Horizontal bars underneath the graphs represent time of treatment, each line represents results from an individual mouse (6-15 mice per group). *P<0.05; ***P<0.001; and ****P<0.0001.

and AML-18 engrafted NSG mice once the disease was established. Two to 3 weeks of the AraC/DNR regimen strongly reduced AML engraftment in all organs (Figure 4A-B). AML-18 cells caused extensive destruction of the

bone marrow space and engraftment data are unavailable. This is consistent with aggressive AML xenografts[32] and was reproducible for AML-5 and AML-18 (*data not shown*). Interestingly, AraC/DNR specifically reduced the absolute
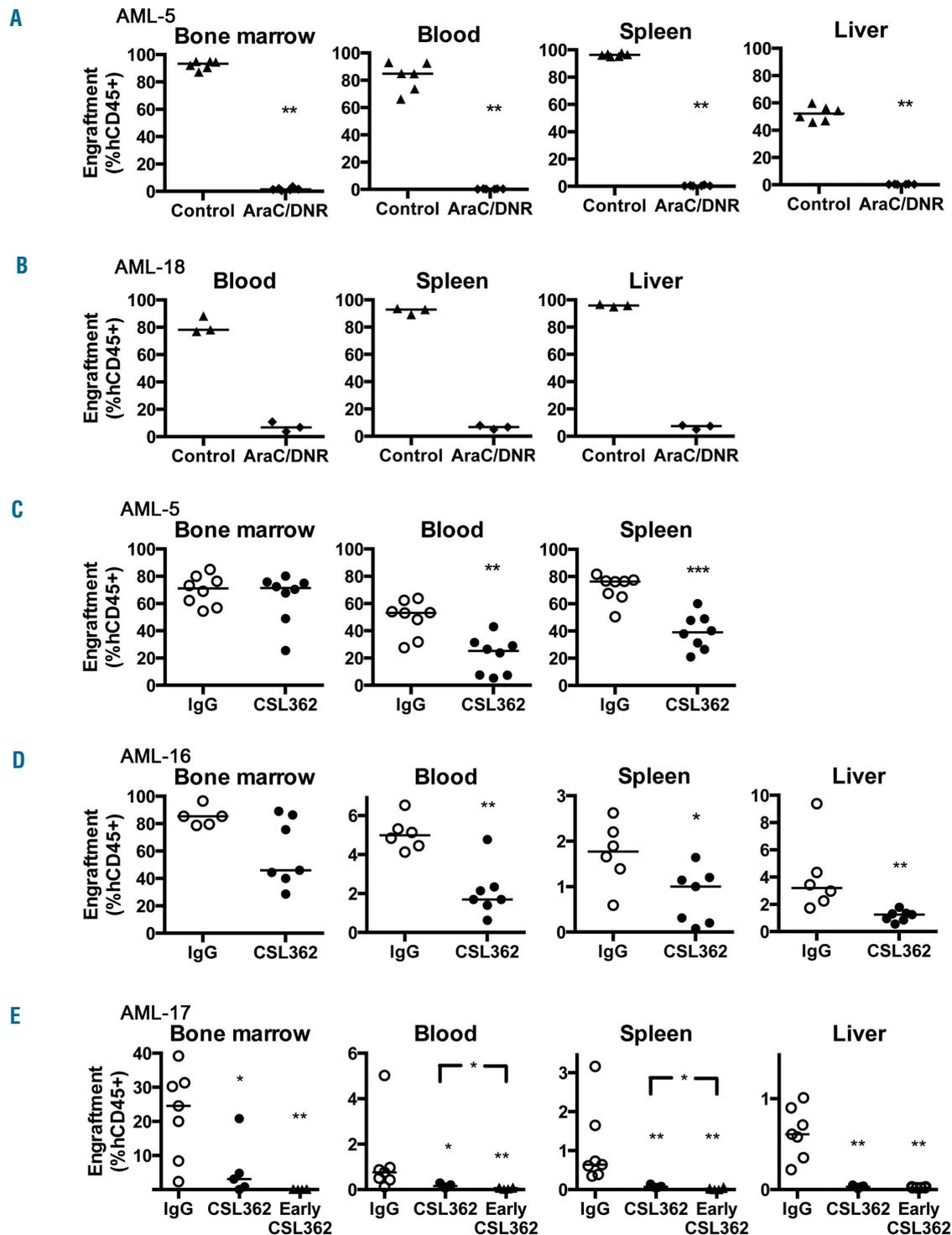
Figure 4. Engraftment levels of AML xenografts *in vivo* in animals treated with the AraC/DNR regimen or CSL362 as monotherapy. (A, B) AML engraftment in various organs of NSG mice treated with AraC (25 mg/kg) and DNR (0.63 mg/kg). (A) For AML-5 engrafted mice, 3 weeks of treatment was initiated on day 14 after-transplantation. (B) For AML-18 engrafted mice treatment was initiated on day 21 after-transplantation and was given for 2 weeks. (C-E) NOD/SCID mice were treated for 3 weeks with CSL362 or isotype control (IgG). For (C) AML-5 and (D) AML-16, treatment was initiated 3 and 6 week after transplantation, respectively. (E) For AML-17 engrafted mice, treatment was initiated either 6 weeks or 5 days (Early CSL362 group) after transplantation. Each point represents an individual mouse (3-8 mice per group), and horizontal bars represent the median. *P<0.05; **P<0.01; and ***P<0.001.

AML-5 blast count, with no effects on the mouse hematopoietic cell count in the bone marrow (*Online Supplementary Figure S7*).

In order for CSL362 to mediate additional clinical benefits against AML, it must demonstrate anti-leukemic effects in the bone marrow and other organs. NOD/SCID mice were used because of their residual immunity when compared with NSG mice.[30] Consistent with this, CSL362 efficacy against AML-5 was attenuated in NSG mice compared with NOD/SCID mice (*Online Supplementary Figure S8*). CSL362 significantly reduced engraftment of AML-5, -16, and -17 in the blood, spleen and liver, but was only significantly effective against AML-17 in the bone marrow (Figure 4C-E). CSL362 was not effective against AML-18 despite initiation of treatments early at 6 days after-transplantation (*Online Supplementary Figure S9*). When CSL362 treatment was initiated early in AML-17 engrafted mice, in a simulation of minimal residual disease, the results tended towards improved efficacy in the bone marrow and other organs (Figure 4E).

In vitro *antibody-dependent cellular cytotoxicity of natural killer cells against acute myelogenous leukemia xenografts with CSL362*

The ability of CSL362 to cause AML xenograft cell lysis *in vitro* in the presence of huNK cells was also tested in ADCC assays. Following 21 days of expansion from donor buffy coats, the average increase in NK cell numbers ranged from 622- to 725-fold at >95% purity with very high expression of CD56, CD16 and positivity for NKp46 (*Online Supplementary Table S2, Online Supplementary Figure S10*). Four separate huNK cell expansions were tested against five AML xenografts, and huNK cells in the absence of CSL362 increased AML cell death by up to 40% (Figure 5A). CSL362-mediated cell death in the presence of huNK cells was significantly increased against AML-5, -9 and -17 compared with isotype controls, with AML-16 also approaching significance (Figure 5A,B).

These results confirm the potency of expanded huNK cells in mediating CSL362-induced cell death.

In vivo *efficacy of CSL362 combined with adoptive transfer of human natural killer cells and chemotherapy*

We next carried out *in vivo* experiments to determine whether adoptive transfer of huNK cells could increase the efficacy of CSL362 against AML xenografts in NSG and NOD/SCID mice. Overall, CSL362 and the addition of huNK cells resulted in no significant delays in leukemia progression against AML-5 (two independent experiments), and AML-17 (*Online Supplementary Figure S11* and *Online Supplementary Table S3*). CFSE-labeled huNK cells were detectable in the peripheral blood, bone marrow, spleen, and liver 1 day after inoculation (*Online Supplementary Figure S12*), indicating that the huNK cells could enter relevant leukemia niches.

CSL362 is currently being tested in AML patients in remission following chemotherapy. This scenario was simulated in the AML xenograft models in two different approaches in which CSL362 and huNK cell treatment was initiated: (i) 1 day after transplantation (Figure 6A-D); and (ii) after the leukemia had been established and subsequently treated with chemotherapy (Figure 6E-H). In both cases, the leukemia burden simulated a minimal residual disease setting. AML-16-engrafted mice were treated for 3 weeks with CSL362 ± huNK cells and this did not delay leukemia progression (Figure 6A-D and *Online Supplementary Table S3*). However, treatment with CSL362 following chemotherapy significantly delayed leukemia progression (P=0.02; Figure 6E-H, *Online Supplementary Table S3*). The addition of huNK cells with CSL362 further delayed AML-16 progression compared with either CSL362 or huNK cells alone following chemotherapy (P=0.0008 and P=0.0004, respectively). A similar experiment was carried out with AML-17, in which the huNK cells were delivered over six doses (*Online Supplementary Figure S13A*) instead of two, to avoid adverse reactions
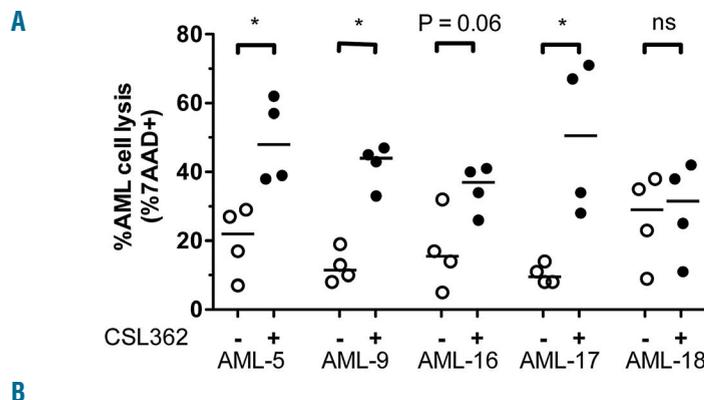
Figure 5. Cytotoxic killing of AML cells by expanded huNK cells and CSL362 in ADCC assays. (A) Cell lysis of AML cells mediated by huNK cells harvested from *ex vivo* expansions, with 10 μg/mL of CSL362 or IgG1 control. Each dot represents an experiment conducted with an independent huNK donor (triplicates were performed per experiment). Background AML cell death was subtracted from all values. Horizontal lines represent the median. *P<0.05; ***P<0.001; and n.s.: non-significant. **(B)** Summary of % cell lysis in ADCC assays with or without CSL362 against AML xenografts with expanded NK cells from different donors, without correction for background AML cell death. Specific lysis refers to the ADCC mediated specifically by the addition of CSL362.

| NK Donor | AML-5 | | | AML-9 | | | AML-16 | | | AML-17 | | | AML-18 | | |
	IgG (%)	CSL 362 (%)	Specific lysis (%)	IgG (%)	CSL 362 (%)	Specific lysis (%)	IgG (%)	CSL 362 (%)	Specific lysis (%)	IgG (%)	CSL 362 (%)	Specific lysis (%)	IgG (%)	CSL 362 (%)	Specific lysis (%)
47008	43	73	30	67	90	23	74	86	12	72	89	17	75	77	2
51691	61	93	32	55	87	32	59	68	9	66	86	20	89	91	2
70382	62	83	21	53	92	39	60	86	26	33	92	59	62	65	3
23561	38	71	33	57	81	24	66	95	29	29	92	63	58	62	4

associated with the combination of large numbers of huNK cells after chemotherapy. CSL362 administration after chemotherapy significantly extended the event-free survival of mice compared with the control (*P*=0.03; *Online Supplementary Figure S13* and *Online Supplementary Table S3*). However, the adoptive transfer of huNK cells was unable to extend the delay in leukemia progression.

The AML-5 xenograft did not develop long-term resistance to CSL362

When CSL362 was tested as a single agent against AML-5-engrafted NOD/SCID mice it produced a leukemia growth delay of 16 days (*P*<0.01, n=6 per group) (Figure 7A,B). The expression of surface CD123 was significantly down-regulated on AML-5 cells in the blood during and

after CSL362 treatments, with some recovery of CD123 expression (Figure 7C) after CSL362 was no longer binding (*data not shown*). These changes in CD123 expression after CSL362 treatments had been observed for other AML xenografts (*data not shown*).

To investigate whether CSL362 exerted selection pressure on AML-5 based on CD123 expression, thus potentially decreasing CSL362 efficacy in regimens with multiple treatment cycles, cells from isotype control (i.e. "CSL362 naïve") and CSL362-treated mice (identified as "CSL362-selected" in Figure 7D-G) were collected from spleens after CSL362 was no longer binding (*data not shown*). These two lines of AML-5 were transplanted into secondary recipient NOD/SCID mice and re-treated with isotype control or CSL362. In mice engrafted with
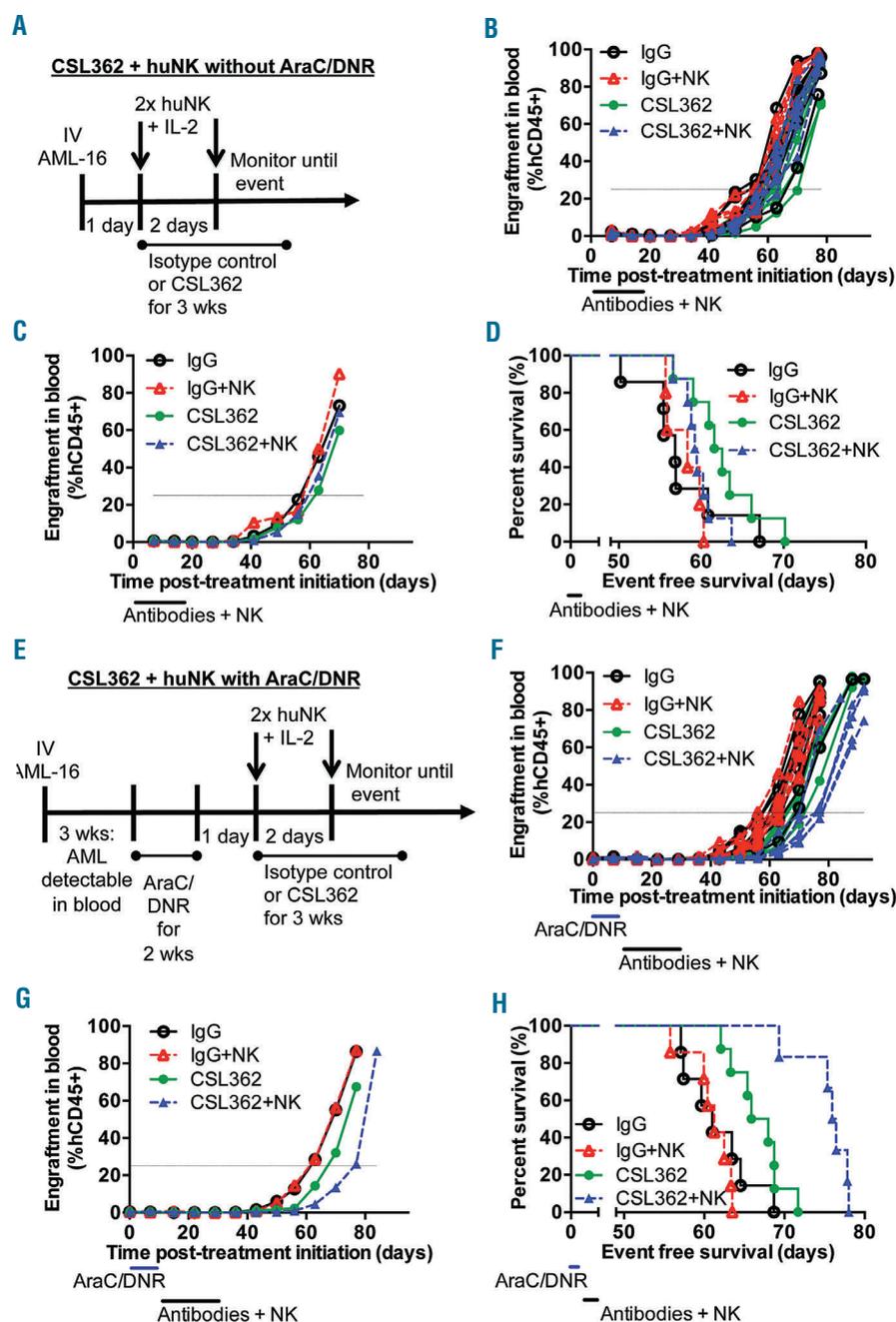
Figure 6. Chemotherapy enhances the efficacy of CSL362 and huNK cell against AML-16 xenografts in NSG mice. (A-D) *In vivo* efficacy of CSL362 and/or adoptive transfer of huNK cells against AML-16 without chemotherapy. (A) Schematic illustrating the treatment of AML-16 engrafted mice without chemotherapy. (B) AML engraftment levels in the blood of individual mice over time. (C) Median engraftment levels in the blood over time. (D) Event-free survival curves. (E-H) *In vivo* efficacy of CSL362 and/or adoptive transfer of huNK cells against AML-16 with chemotherapy. (E) Schematic illustrating the treatment of AML-16 engrafted mice after chemotherapy. (F) AML engraftment levels in the blood of individual mice over time. (G) Median engraftment levels in the blood over time. (H) Event-free survival curves. Blue bars mark the time of treatment with AraC/DNR (25 mg/kg AraC, and 0.63 mg/kg DNR); black bars mark the time of treatment with antibodies (300 µg/dose, three times per week) and huNK cells (20 x 10⁶ huNK cells, 500 IU interleukin-2 per injection). Five to eight mice per group.

"CSL362 naïve" and "CSL362-selected" AML-5 cells the leukemia growth delay was 5.5 days (P<0.0001, n=7-8 per group) and 5.2 days (P<0.0001, n=8 per group), respectively, when compared with their corresponding isotype controls (Figure 7D-E). CD123 expression was again consistently and statistically lower for CSL362-treated mice, regardless of the history of the cells transplanted (Figure 7F, P<0.001 when comparing CSL362-treated mice with their respective controls at all time-points before day 67, n=4-8). CD123 expression on AML-5 cells in CSL362-treated mice recovered to the same level as that in isotype controls 25 days after the last CSL362 injection (Figure 7G, P>0.33 between isotype control and CSL362-treated

groups, n=4-8). These results suggest that AML-5 xenografts did not develop resistance to CSL362.

Discussion

In this study, we developed a panel of continuous AML xenografts in immune-deficient mice that produced clinically relevant disease pathology, exhibited reproducible engraftment, and responses to an induction-type AraC/DNR chemotherapy regimen that allowed combination testing with novel treatments such as CSL362. Continuous AML xenografts engrafted more rapidly and
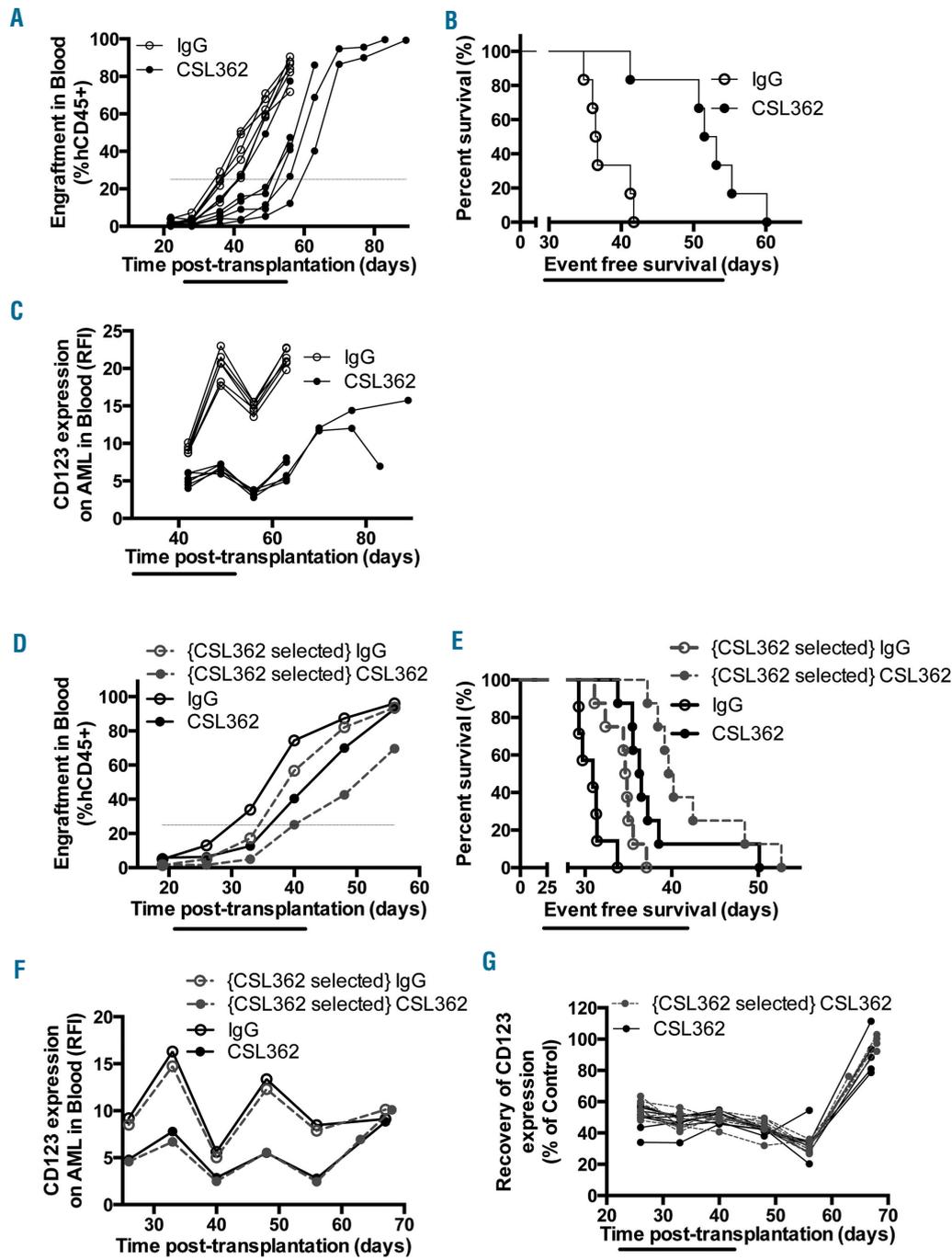
Figure 7. CSL362 treatment does not select for subsequent resistance. (A) Engraftment levels in the blood of individual AML-5 engrafted mice over time and (B) event-free survival curves after treatment with isotype control or CSL362. (C) Changes in surface CD123 expression on AML-5 cells in the blood of mice treated with isotype control or CSL362. (D-G) AML-5 cells were harvested from mice treated with isotype control or CSL362 (identified as "CSL362-selected" and transplanted into secondary recipient mice, then further treated with isotype control or CSL362. (D) Median AML-5 engraftment levels (7-8 mice per group) and (E) Event-free survival curves in secondary recipient mice. (F) Changes in surface CD123 expression levels on AML-5 cells in the blood of secondary recipient mice during and after treatment. (G) Recovery of surface CD123 expression levels as a % of respective isotype control on AML-5 cells in the blood of CSL362-treated mice engrafted with CSL362-selected or naïve AML-5 cells.

reproducibly in later passages, suggesting selection of AML subclone(s), Specific evidence for clonal selection upon xenografting was obtained by molecular analysis of candidate genes. In particular to *FLT*3-ITD, loss of a minor subclone (AML-5), selection of a dominant subclone from multiple subclones present at diagnosis (AML-9 and AML-16), emergence of a new subclone (AML-17), and increased mutant levels (AML-9 and AML-16) are all consistent with disease evolution from diagnosis to relapse.[33-35] These observations suggest that the xenograft model selects for more aggressive, self-supporting clones of the disease.

AML xenograft cells also displayed different immunophenotypes depending on the organ of infiltration. While the relevance of this observation is not clear it highlights the potential of this model to examine subtle microenvironment-leukemia interactions. The mechanism of efficacy of novel compounds is often not fully understood during pre-clinical development, and subtle regimen changes such as providing an inhibitor before, during or after chemotherapy can increase efficacy.[36] The AML xenograft models with AraC/DNR treatments provide a system for optimizing a regimen before a clinical trial. In addition, AML xenografts with specific mutations or karyotypes can be used to support pre-clinical development of specific agents.

In AML patients, AraC and DNR are administered at doses from 100 and 45 mg/m^2, respectively.[37] The body surface area-normalized doses of AraC and DNR used in our xenograft studies were 75 and 1.9 mg/m^2, respectively.[38] While the dosage of AraC can be increased in mice to the human equivalent, anthracyclines are substantially more toxic in mice.[39,40] The AraC/DNR regimen developed in this study produced mild and temporary side effects only, thus allowing novel agents to be combined for investigation. The regimen of AraC/DNR used was sufficient to produce remission-like conditions in the bone marrow and relevant organs in engrafted mice.

Surprisingly, this study discovered that irradiation of NSG mice impaired the engraftment of three of three established xenografts tested, in contrast to the situation with NOD/SCID mice. We reason that whole body irradiation of NSG mice may have a negative impact on the murine microenvironment that supports AML homing and engraftment. Sub-lethal irradiation of mice can be associated with adverse events, and increased toxicity of chemotherapy.[39] This discovery provides a more robust and reliable model for pre-clinical testing of novel therapies.

Previous results showed that the Fc region of the anti-CD123 antibody 7G3 was important in mediating efficacy[8] and huNK cells were primarily responsible for this effect.[15] This is supported by the decrease in CSL362 efficacy against the AML-5 xenograft in NSG mice when compared with NOD/SCID mice. The *in vitro* ADCC assays verified the activity of expanded huNK cells and their role in mediating CSL362-directed anti-leukemic effects. Interestingly, huNK cells alone were able to kill AML-18 cells *in vitro* but the addition of CSL362 had no further effect; future examination of AML-18 and other AML samples might reveal biomarkers predictive of CSL362 responses. We attempted to complement the lack of functional immune cells in NOD/SCID and NSG mice[30,31,41] by adoptive transfer of huNK cells, but this did not consistently improve *in vivo* efficacy of CSL362. This results was similar to that of a clinical study in which patients with metastatic melanoma or renal cell carcinoma received large doses of huNK cells with no clear benefit.[42] More sophisticated methodology, such as the transplantation of fetal thymic, liver tissue with hematopoietic stem cells into NSG mice, will produce a more functional human immune system,[43] although it will be technically challenging to incorporate AML xenografts into such a humanized model. Despite the relative lack of immunity in NOD/SCID mice, CSL362 significantly reduced the leukemic burden in the peripheral blood, spleens and livers of three out of four AML xenografts tested, but only significantly decreased bone marrow disease in one out of four xenografts. It is unclear whether the process of establishing continuous AML xenografts affected the individual responses of these AML cells to CSL362. Moreover, we cannot exclude the possibility that the results obtained may have been influenced by the aggressive nature of the primary AML samples studied.

To assess CSL362 side effects, cynomolgus monkeys (n=12) were treated with a single dose of CSL362 (0.01-10 mg/kg). CD123 expressing basophils and dendritic cells were depleted within 6 h and subsequently recovered 4 weeks after treatment with no other significant side effects.[15] Furthermore, our model indicated that AML cells do not develop long-term resistance to CSL362. These findings strongly support further examination of CSL362 in the current clinical trial in remission AML patients.

In this study we showed that the %hCD45$^+$ cells in the murine peripheral blood provided a surrogate indicator of overall disease burden in the animals, and that the disease would result in mortality if allowed to progress. It is, therefore, important to acknowledge that measurements of mouse event-free survival in all efficacy experiments used a pre-defined endpoint of 25% hCD45$^+$ cells in the peripheral blood or an animal exhibiting signs of leukemia-related morbidity, and event-free survival data should be interpreted accordingly.

For some AML xenografts, CSL362 alone did not improve mouse event-free survival. However, when treatments were administered after chemotherapy, CSL362 prolonged event-free survival to a modest extent for AML-16- and AML-17-engrafted mice. Bone marrow mesenchymal stem cells were reported to suppress the activity of NK cells against K562 cells in a mouse model[44] and the chemotherapy in the present study may have affected these bone marrow cells, decreasing immunosuppression. Alternatively, since AraC treatment in pediatric patients increased levels of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6 and interferon-γ,[45] and similarly, doxorubicin increased the levels of interleukin-1β in mice[46] and interleukin-1β, tumor necrosis factor-α and interleukin-6 in murine macrophages *in vitro*, the chemotherapy in this study may have generated a pro-inflammatory environment that potentiated the effects of the huNK cells and CSL362. Interestingly, the addition of huNK cells to CSL362 after chemotherapy prolonged the event-free survival of AML-16, but not of AML-17-engrafted mice. This result may reflect the ability of different AML to interact with the microenvironment to induce immunosuppression, or cytoprotection since the huNK cells were able to lyse a substantial proportion of AML-17 cells *in vitro*. HuNK cell adoptive transfer has been investigated in human patients with limited survival benefits.[42,47] The use of antibody with NK cells increased ADCC or activated NK cells,[48,49] and our current results supported this, but the mechanism responsible for the anti-leukemic

effects of the CSL362 and huNK combination after chemotherapy, and the role of the microenvironment require further elucidation.

In conclusion, this AML xenograft model provides a platform for the testing of new therapeutics and combinations, and is especially crucial for heterogeneous diseases such as AML, in which the probability of developing resistance to a single agent is high.[50] The combination of chemotherapy, CSL362 and huNK cell adoptive transfer is novel and produced some evidence of *in vivo* efficacy; CSL362 will be further examined in the current phase I clinical trial.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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