Targeting CD70 with cusatuzumab eliminates acute myeloid leukemia stem cells in patients treated with hypomethylating agents

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; AML, Acute myeloid leukemia; CR, complete remission; CRi, complete remission with incomplete hematologic recovery; ELDA, enhanced limiting dilution analysis; HMA, hypomethylating agents; HSCs, hematopoietic stem cells; LSCs, leukemia stem cells; LSPCs, leukemia stem and progenitor cells; mAb, monoclonal antibody; MRD; minimal residual disease; PB, peripheral blood; PDX, patient-derived AML xenograft experiments; PR, partial remission; sCD27, soluble CD27; scRNA-seq, single-cell RNAseq analysis; TNF, tumor necrosis factor.

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Acute myeloid leukemia (AML) is driven by leukemia stem cells (LSCs) that resist conventional chemotherapy and are the major cause of relapse[1,2]. Hypomethylating agents (HMA) are the standard of care in the treatment of elderly or unfit AML patients, but responses are modest and not durable[3–5]. Here, we demonstrate that LSCs upregulate the tumor necrosis factor (TNF) family ligand CD70 in response to HMA treatment resulting in increased CD70/CD27-signaling. Blocking CD70/CD27-signaling and targeting CD70-expressing LSCs with cusatuzumab, a human αCD70 monoclonal antibody (mAb) with enhanced antibody-dependent cellular cytotoxicity activity (ADCC), eliminated LSCs in vitro and in xenotransplantation experiments. Based on these pre-clinical results, we performed a phase 1/2 trial in previously untreated elderly AML patients with a single dose of cusatuzumab monotherapy followed by a combination therapy with the HMA azacitidine (NCT03030612). Here, we report results from the phase 1 dose escalation part of the clinical trial. Hematologic responses in the 12 patients enrolled included 8 CR, 2 CRi, and 2 PR with 4 patients achieving MRD negativity by flow cytometry at $<10^{-3}$. Median time to response was 3.3 months. Median progression free survival (PFS) was not reached yet at the time of the data cut-off. No dose-limiting toxicities (DLTs) were reported and the maximum tolerated dose (MTD) of cusatuzumab was not reached. Importantly, cusatuzumab treatment significantly reduced LSCs and triggered gene signatures related to myeloid differentiation and apoptosis.

The TNF receptor ligand CD70 is transiently up-regulated on immune cells upon activation but not expressed in normal tissue and on hematopoietic cells during homeostasis[6,7]. However, CD70 is expressed on various solid tumors and on Non-Hodgkin lymphomas and its expression correlates with poor survival[8–10]. We recently demonstrated that $CD34^+$ AML cells (progenitors and LSCs) consistently express CD70 as well as its receptor CD27 and that cell-autonomous CD70/CD27-signaling propagates the disease[11]. The promoter of CD70 is

sensitive to methylation[11,12]. To analyze whether HMA treatment results in up-regulation of CD70 on LSCs, BM lin$^-$CD90$^-$CD34$^+$CD38$^-$ LSCs[1] from newly diagnosed AML patients (Supplementary Table 1, Supplementary Fig. 1a) were cultured in the presence and absence of a pharmacological concentration of decitabine (D) or azacitidine (A)[13,14]. HMA treatment reduced LSC numbers by approximately 45%, independent of the risk category[15] (Supplementary Fig. 1b). The HMA-resistant LSCs had a significantly higher expression of CD70 than vehicle-treated control samples (Figs. 1a, Supplementary Figs. 1c-d). In contrast, HMA treatment reduced the numbers of lin$^-$CD90$^-$CD34$^+$CD38$^+$ blasts and lin$^-$CD90$^+$CD34$^+$CD38$^-$ hematopoietic stem cells (HSCs) from normal control BM (ctrl.) without increasing CD70 expression (Figs. 1a, Supplementary Figs. 1b, d, e). Similarly, HMA treatment did not affect CD70 expression on NK cells and DCs derived from peripheral blood (PB) of healthy donors, two cell populations with reported transient CD70 expression during activation (Supplementary Figs. 1f, g)[7].

HMA treatment increased CD70 expression on LSCs but not lymphocytes in PB of AML patients (Fig. 1b, c, Supplementary Fig. 1h). CD70 expression on AML LSCs negatively correlated with the degree of methylation of the CD70 promoter (Fig. 1d). In contrast, the CD70 promoter of HSCs showed only a moderate degree of methylation (Supplementary Fig. 1i). Treatment of lin$^-$CD90$^-$CD34$^+$AML cells in vitro with HMA significantly reduced the methylation of the CD70 promotor (Fig. 1e). The CD70 promotor contains binding sites for various transcription factors such as specificity protein 1 (SP-1)[16]. *SP-1* expression was up-regulated in lin$^-$CD90$^-$CD34$^+$AML cells after treatment with HMAs, whereas *miR-29b,* a negative regulator of *SP-1*[17], was expressed at lower levels (Figs. 1f, g). Overall these data indicate that HMAs induce CD70 expression in leukemia stem/progenitor cells (LSPCs) by demethylation of the CD70 promotor and by down-regulating *miR-29b* levels resulting in the up-regulation of the transcription factor SP-1.

Ligation of CD27 on LSCs by CD70 induces Wnt-pathway activation, symmetric cell division and thereby maintains and expands LSCs[11]. To determine the functional relevance of the HMA-induced up-regulation of CD70 and increased CD70/CD27-signaling, we cultured LSCs in the presence of either a blocking αCD70 mAb (clone: 41D12-D)[18,19] or HMA alone or in combination (αCD70/HMA). 41D12-D is an αCD70 mAb with deficiency in effector function due to E233P/L234V/L235A amino acid substitutions in the CH2 region[18,19] and has the same binding domain as cusatuzumab. CD27 is shed from the cell surface after ligation with CD70 resulting in the release of soluble CD27 (sCD27)[7]. Treatment of LSCs with αCD70 mAb in vitro significantly reduced sCD27 levels suggesting that the CD70/CD27 interaction is efficiently blocked (Fig. 1h). HMA treatment of LSCs strongly increased sCD27 levels indicating enhanced CD70/CD27-signaling. This increased CD27 ligation and release of sCD27 was completely blocked by adding αCD70 mAb (Fig. 1h). αCD70 treatment inhibited cell growth without affecting cell viability. In contrast, HMA treatment triggered cell death of LSCs (Supplementary Figs. 1j, k). Importantly, the αCD70/HMA combination treatment significantly reduced LSC numbers compared to monotherapy by increasing cell death (Supplementary Fig. 1j).

As previously reported[11], treatment with an αCD70 mAb reduced colony formation and induced differentiation of LSPCs as indicated by a trend towards increased cell numbers per colony (Fig. 1i, Supplementary Fig. 1l). The combination treatment synergistically reduced colony formation capacity compared to monotherapy (Fig. 1i). HMA treatment of LSCs prior to plating in methylcellulose up-regulated CD70 mRNA expression independent of the addition of αCD70 mAb (Supplementary Fig. 1m). The impaired colony formation after combination treatment observed after the first plating was maintained in subsequent re-platings in the absence of αCD70 mAb and HMA (Fig. 1i). These experiments suggest that the αCD70/HMA combination strongly reduces LSCs in vitro. The clonogenic potential of

HSCs was only affected to a minor extent by HMA treatment but not by the addition of the αCD70 mAb (Supplementary Fig. 1n) and CD70 mRNA expression in HSCs was not affected by HMA treatment prior to plating in methylcellulose (Supplementary Fig. 1o). These experiments indicated that blocking the CD70/CD27 interaction in combination with HMA treatment synergistically eliminates LSPCs in vitro.

To analyze the therapeutic potential of combining a blocking αCD70 mAb with HMAs in vivo, we performed patient-derived AML xenograft experiments (PDX)[1]. After engraftment of human AML cells, NOD/SCID $\gamma c^{-/-}$(NSG) mice were randomized to treatment with vehicle (Veh), αCD70 mAb (41D12-D, 3x10mg/kg over 5 days), decitabine (D, 1.5mg/kg/day) or αCD70 mAb and decitabine in combination (Supplementary Fig. 2a). Decitabine treatment induced a significant up-regulation of CD70 on LSCs but not on blasts (Figs. 1j, Supplementary Fig. 2b-d). Furthermore, sCD27 levels were elevated in sera of PDX AML mice treated with decitabine compared to controls, indicating that decitabine-induced CD70 expression triggers CD70/CD27-signaling in AML mice *in vivo* (Fig. 1k). Treatment with the αCD70 mAb significantly reduced sCD27 levels especially in αCD70/decitabine-treated mice (Fig. 1k).

αCD70 and decitabine monotherapy significantly reduced leukemic engraftment in BM, spleen and blood compared to vehicle-treated AML mice (Supplementary Figs. 2e, f, data not shown). Importantly, co-treatment synergistically reduced the engraftment of $CD45^+lin^-CD34^-$ and $CD45^+lin^-CD34^+$ AML cells as well as of $CD45^+lin^-CD34^+CD38^-$ LSCs in the BM (Figs. 1l, m, Supplementary Figs. 2g, h). Similarly, the more primitive CD45RA-expressing LSCs[20] are reduced after combination treatment (Supplementary Fig. 2i). Decitabine monotherapy had no effect on LSC numbers (Fig. 1m, Supplementary Fig. 2h). BM cells from αCD70/D-treated mice formed significantly fewer colonies in methylcellulose compared to either monotherapy or untreated controls (Fig. 1n), indicating that AML LSPCs were reduced

by αCD70/D-treatment. Enhanced limiting dilution analysis (ELDA)[21] from the BM of primary PDX AML mice to NSG mice revealed that αCD70/D co-treatment significantly reduced human AML LSCs in PDX mice compared to either monotherapy as indicated by a substantial reduction in LSC frequency by a factor of 2.9 and 11 in patients P10 and P25, respectively (Fig. 1o, Supplementary Table 2). The PDX results indicated that HMA treatment increased CD70 expression and promoted consecutive CD70/CD27-signaling. The combination with blocking αCD70 mAb synergistically reduced LSC numbers in vivo.

Next, we analyzed whether targeting CD70 with the ADCC-enhanced αCD70 mAb cusatuzumab efficiently reduces LSPCs than the blocking αCD70 mAb (41D12-D). In the absence of NK cells cusatuzumab demonstrated a similar capacity in reducing colony formation in vitro as the blocking αCD70 mAb. In the presence of NK cells, cusatuzumab but not 41D12-D mAb treatment further reduced colony formation (Fig. 2a). In contrast, cusatuzumab did not affect colony formation of HSCs from normal control BM (Fig. 2b). To validate our findings in vivo, PDX AML mice were treated either with control mAb, αCD70 mAb (clone: 41D12-D) or cusatuzumab. Cusatuzumab was similarly effective in reducing leukemia cell engraftment and LSC numbers as the blocking αCD70 mAb in the absence of NK cells. However, in the presence of NK cells, cusatuzumab further reduced leukemia engraftment and LSC numbers in BM and spleen (Figs. 2d-e, Supplementary Figs. 3a-c). The reduction in LSPC numbers in the BM was functionally confirmed by colony forming assays ex vivo (Fig. 2f).

CD70 up-regulation by HMA may render LSCs even more susceptible to direct cytolytic interventions. To test this hypothesis, we performed a drug combination study according to the Chou-Talalay method[22]. Decitabine increased CD70 expression and in combination with cusatuzumab in the presence of NK cells synergistically killed CD70-expressing MOLM-13 cells and NOMO-1 cells in a broad dose range (Supplementary Figs. 4a-i).

Next, we treated human LSCs and blasts with cusatuzumab or decitabine monotherapy or in combination and assessed colony formation. Although, AML blasts do not upregulate CD70 upon treatment with HMA (Supplementary Fig. 1e), CD70 is expressed on both LSCs$^-$ and on blasts[11]. Indeed, cusatuzumab/HMA co-treatment in the presence of NK cells effectively eliminated both, LSCs and blasts, compared to all other treatment groups (Fig. 2g, h). Decitabine monotherapy quite efficiently eliminated blasts but only marginally reduced LSCs. Only the combination with cusatuzumab eliminated LSCs. Cusatuzumab treatment uniformly promoted the expression of myeloid differentiation genes such as SPI1 (PU.1) and CEBPα and reduced cell viability in average by approximately 85 percent compared to control treatment (Figs. 2i, j).

These results indicate that a combination of HMA with the ADCC-enhanced αCD70 mAb cusatuzumab eliminates LSCs synergistically and more efficiently than in combination with an αCD70-blocking mAb.

Based on our preclinical data, we designed a phase 1/2 trial to study safety, tolerability and the efficacy of cusatuzumab as monotherapy and in combination with azacitidine (EudraCT number 2016-002151-17; see online methods). Untreated AML patients, including *de novo*, secondary and therapy-related AML, not fit for intensive chemotherapy were included in the study. Azacitidine was administered at a standard dose of 75mg/m^2 subcutaneously (s.c.) for seven consecutive days every 28 days. Cusatuzumab was infused at day 3 when CD70 was upregulated on LSCs by HMA and on day 17 of each treatment cycle. A first infusion of cusatuzumab at day -14, in the absence of azacitidine, enabled us to study the effect of cusatuzumab monotherapy (Fig. 3a).

The clinical cut-off for data from the phase 1 part of the study was February 18, 2019 with the primary objective to determine the maximum tolerated dose (MTD) of cusatuzumab and the

recommended dose for the phase 2 part (RP2D) in combination with azacitidine. Twelve untreated AML patients with a median age of 75 years (range, 64-84) were enrolled in four sequential dose cohorts of cusatuzumab (1, 3, 10, or 20mg/kg; 3 patients per dose cohort) in the dose-escalation part of the trial (Fig. 3a, Supplementary Table 3). Patients had adverse (n=5), intermediate (n=5) and favorable (n=2) ELN risk features based on genotype and cytogenetics[15]. No dose-limiting toxicities (DLTs) were reported and the MTD of cusatuzumab was not reached.

All 12 patients had at least one treatment-emergent adverse event (TEAE, total of 167 events), and all 12 patients had at least one grade ≥3 TEAEs (71 events). Nine patients were reported with drug-related TEAEs (17 events) of which two patients experienced infusion-related reactions (IRR, six events). Treatment-emergent serious AEs (SAE) were reported for nine patients (19 events) out of which two were drug-related SAEs reported for one single patient (Supplementary Tables 4-6).

Hematologic toxicities related to azacitidine were the most frequent TEAE (34 events in 10 patients)[3]. The only patient with early death succumbed to progression of AML. In addition, no increase of viral or fungal infections was observed.

Cusatuzumab monotherapy did not affect the numbers of $CD4^+$ and $CD8^+$ T cells in PB, two cell populations which are known to transiently express CD70 during immune activation[7]. In contrast, the $CD4^+/CD8^+$ T cells ratio slightly increased after cusatuzumab monotherapy (Supplementary Fig. 5). Long-term exposure of the patients to the cusatuzumab/azacitidine combination treatment did not affect normal hematopoiesis as indicated by normal blood counts and leukocyte differential in the six patients which have been treated for longer than 6 months at the time point of data cut-off February 18th, 2019 (Supplementary Table 7).

In the phase 1 part of the clinical trial, best hematologic response (BR) was complete remission (CR) in 8 patients, CR with incomplete hematologic recovery (CRi) in 2 patients, and partial remission (PR) in 2 patients (Fig. 3b, c, Supplementary Table 8). Thus, 10 out of

12 patients achieved a CR/CRi. Responses were observed at all dose levels of cusatuzumab and median time to response was 3.3 months. Importantly, responses were durable with 6 patients still on study treatment at the time of data cut-off and median progression free survival (PFS) was not reached yet. Four out of 9 evaluable patients (44%) with CR/CRi achieved MRD-negativity by flow cytometry in the BM at a threshold of 10^{-3} (Fig. 3d, Supplementary Table 8). End of treatment (EOT) was caused by progression in 4 patients, by referral to allogeneic transplantation in CR in patient C2 and by treatment-related toxicity (hypertension) in 1 patient.

Importantly, cusatuzumab monotherapy reduced BM blasts in only two weeks in all patients on average to 32% with three responders (1 CR, 1 CRi and 1 PR) among the 12 patients. The combination with azacitidine further reduced the BM blast counts by 92% compared to C1D1 (Fig. 3e-f). No correlation between response to cusatuzumab treatment with the numbers and frequencies of NK cells in peripheral blood at day -14 could be observed (Supplementary Fig. 6).

CD70 expression was detectable on $CD34^-$ AML cells and LSPCs in a majority of patients (Fig. 4a). To monitor the degree of CD70/CD27 interaction in the patients in vivo, we assessed sCD27 levels in the sera of patients enrolled in the study at day -14. sCD27 levels were significantly increased in all AML patients compared to healthy age-matched controls (HD, Fig. 4b). Cusatuzumab monotherapy significantly reduced sCD27 levels within 14 days of treatment (Fig. 4b, c). The combination of azacitidine with cusatuzumab further decreased sCD27 at the time point of BR to levels comparable to HD controls (Fig. 4c, d).

Cusatuzumab monotherapy reduced LSPC numbers by a factor of 3, as assessed by the colony forming capacity of plated total BM cells (Fig. 4e). ELDA revealed that monotherapy with cusatuzumab significantly reduced LSC numbers compared to day -14 in all patients analyzed (Figs. 4f-h).

The in vitro experiments do not allow to unambiguously distinguish between LSPCs and normal hematopoietic stem/progenitor cells. The most stringent experiment to assess LSC numbers is to analyze engraftment of titrated numbers of BM cells after transplantation into sub-lethally irradiated NSG mice (Fig. 4i). ELDA indicated that cusatuzumab monotherapy in patient C2 reduced the LSC frequency by approximately 47-fold (Fig. 4j, k). These data suggest that targeting CD70 by cusatuzumab monotherapy reduces LSCs in AML patients.

To address the molecular mechanism of cusatuzumab on AML LSCs, we performed scRNA-sequencing of FACS-purified lin^-CD34^+ AML cells from patients C8 and C10 at day -14 and after cusatuzumab monotherapy (C1D1) (Supplementary Figs. 7a-c). Patient C10 harbored two AML clones which differed in the expression of CD34; the $CD34^+$ clone was purified and included in the scRNA-sequencing analysis (Supplementary Table 3). To focus on malignant cells, we took advantage of the molecular mutations identified at diagnosis in patients C8 (DNMT3a) and C10 (ASXL1, EZH2, RUNX1, SH2B3, ZRSR2) using a data crushing approach separating cells that did or did not express the molecular marker. This approach identified a frequency of 77% and 74% of malignant AML cells at day -14 and C1D1 for patient C8, respectively, and 99.9% for patient C10 at both time points. No distinct clusters could be identified in principal component analysis (PCA) of single cells of patients C8 and C10 at diagnosis or after cusatuzumab monotherapy suggesting the predominance of a clonal population with similar cellular states (Supplementary Fig. 7d). We identified 47 and 18 differentially expressed genes after cusatuzumab monotherapy for patients C8 and C10, respectively (Supplementary Figs. 7e-h, Supplementary Tables 9, 10). Patients C8 and C10 shared 9 differentially expressed genes (7 mRNAs and 2 LncRNAs, Fig. 4l). Gene ontology analysis of these 9 genes revealed a preferential involvement in TGF-β, AP-1-, MAPK-, cAMP-, TNF-, MyD88-signaling and induction of pro-inflammatory cytokines all associated with myeloid cell differentiation and/or apoptosis (Fig. 4m).

Lastly, we assessed the level of apoptotic lin^-CD34^+ LSPCs at day -14 and C1D1 in 7 out of 12 patients by Annexin-V staining. After cusatuzumab treatment (C1D1), the frequency of apoptotic cells was significantly increased compared to day -14 (Fig. 4n). Collectively, these data suggest that cusatuzumab treatment triggers differentiation and apoptosis in LSPCs.

The CD70/CD27 interaction maintains self-renewal of AML LSCs by activating Wnt-signaling and by promoting symmetrical cell division[11]. Since CD70 is only transiently expressed on activated lymphocytes but not during homeostasis[7], targeting the CD70/CD27 interaction may allow selective elimination of CD70-expressing LSCs. In the present study, we document CD70 up-regulation and reinforced CD70/CD27-signaling on LSCs in response to HMA treatment. Increased expression of CD70 offers two therapeutic strategies to selectively target LSCs. First, blocking the CD70/CD27 interaction reduces CD27/TNIK/Wnt-signaling and self-renewal of LSCs and induces differentiation[11]. Secondly, the unique expression pattern of CD70 allows for direct cytolytic targeting[11,23]. We explored both possibilities in vitro and in a limited number of xenotransplantation experiments using an αCD70 mAb with deficiency in effector function due to E233P/L234V/L235A amino acid substitutions in the CH2 region[18,19] and an ADCC-enhanced version of the same clone (cusatuzumab)[19,24]. Both, blocking CD70/CD27-signaling and ADCC-mediated target cell killing synergistically eliminated LSCs. However, cusatuzumab was clearly superior to the blocking-only variant of the antibody. Therefore, cusatuzumab was tested in a phase 1/2 trial together with azacitidine. Within only two weeks, cusatuzumab monotherapy reduced blast counts in the BM on average to 32% with two patients reaching a CR (1 CR, 1 CRi) and one patient a PR. In addition, cusatuzumab monotherapy significantly reduced LSCs up to 50-fold.

Overall response rate for the combination of cusatuzumab with azacitidine was 100% (8 CR, 2 CRi, 2 PR) with 44% of patients with CR/CRi achieving flow MRD-negativity in BM.

296 Responses were observed in all dose levels of cusatuzumab. Although this phase 1 study only
297 included 12 patients, the observed response rate favorably compares with historical data on
298 HMA monotherapy[3,4].
299 Different novel compounds have been tested in combination with low-dose Ara-C or HMAs
300 in elderly non-fit AML patients (reviewed in [25]). Similar to cusatuzumab, venetoclax
301 eliminates LSCs, albeit by a different mechanism, i.e. by suppressing oxidative
302 phosphorylation[26].
303 In conclusion, cusatuzumab monotherapy and in combination with azacitidine is highly active
304 previously untreated patients with AML unfit for intensive chemotherapy. Cusatuzumab
305 eliminates CD70-expressing LSCs potentially leading to deep and durable remissions.
306 Currently ongoing and future clinical phase 2 and 3 studies will further investigate the
307 potential of cusatuzumab to induce durable responses and deep remissions in combination
308 with HMA in more patients.

Acknowledegments

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Competing interests statement

C.R and A.F.O. are listed as investor on a patent held by the University of Bern on targeting CD70 for the treatment of AML.

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Figure legends

Fig. 1: αCD70/decitabine combination therapy eradicates human CD34$^+$CD38$^-$ LSCs in vitro and in vivo. (a) 10^5 FACS-purified lin$^-$CD90$^-$CD34$^+$CD38$^-$ LSCs from BM of newly diagnosed AML patients and lin$^-$CD90$^+$CD34$^+$CD38$^-$ HSCs from normal donor BM (ctrl., Supplementary Table 1) were cultured in StemSpan SFEM medium in the presence or absence of 0.5 µM azacitidine (A, P8), decitabine (D, P11) or vehicle (Veh) in duplicates. Fold change ΔMFI CD70 after HMA treatment (AML is depicted as open symbols, ctrl. as closed symbols, azacitidine (A) in red and decitabine (D) in blue; n=3 and 16 AML patients for A and D, respectively, and n=3 per HMA for ctrl.). Significance was determined using a

two-sided Student's t-test (ALL vs ctrl.). **(b)** Representative FACS plots of CD70 expression on lin$^-$CD90$^-$CD34$^+$CD38$^-$ LSC in the PB of AML patient P22 at diagnosis and after 1 cycle of azacitidine or decitabine treatment A(7), 75 mg/m^2, daily for 7 days, P22). Isotype is depicted in grey; CD70 staining in black at diagnosis and in red after treatment with azacitidine. ΔMFI: MFI staining - MFI isotype. Experiment was performed once for each patient depicted. **(c)** Fold change ΔMFI CD70 (Decitabine, 20 mg/kg, daily for 5 days (D (5)) vs. diagnosis; A (7) vs. diagnosis (AML is depicted as open symbols, azacitidine (A) in red and decitabine (D) in blue) on lin$^-$CD90$^-$CD34$^+$CD38$^-$ AML LSC and lymphocytes. (n=3 and 6 AML patients for A and D, respectively). Significance was determined using a two-sided paired t-test. **(d)** Correlation of methylation state at the *SP-1* binding site of the CD70 promoter in lin$^-$CD90$^-$CD34$^+$CD38$^-$AML LSCs vs. CD70 protein expression (ΔMFI CD70) in newly diagnosed AML patients (n=10). ΔMFI: MFI staining - MFI isotype. The correlation coefficient r was determined using a Pearson correlation. Significance was determined using a two-sided t-test. **(e-g)** lin$^-$CD90$^-$CD34$^+$ AML cells were cultured in the presence of the HMA decitabine (e) and decitabine or azacitidine (f, g) in triplicates as described in (a) and **(e)** methylation state of the CD70 promoter (n=5 AML patients) , **(f)** *SP*-1 and **(g)** *miR-29b* expression was assessed 48h later (n=3 and 6 AML patients for A and D, respectively). Fold change is indicated as A vs. Veh or D vs. Veh. Significance in **(e)** was determined using a two-sided paired t-test. Significances for panel **f, g** were determined using a two-sided one-sample tests (hypothetical value =1**; f,** fav: $P = 0.01303$; int: $P = 0.0079$; adv: $P = 0.0205$. **g,** fav: $P = 0.0133$; int: $P = 0.0178$; adv: $P = 0.0127$.) **(h, i)** FACS-purified lin$^-$CD90$^-$CD34$^+$CD38$^-$ LSCs from BM of newly diagnosed AML patients were cultured in the presence or absence of 0.5μM of HMA (AML is depicted as open symbols, azacitidine (A) in red and decitabine (D) in blue) or αCD70 mAb (αCD70, clone 41D12-D, 10μg/ml) alone or in combination in triplicates for 72h (h) or overnight followed by plating into methylcellulose

containing αCD70 and HMA or both. Colonies and cells were enumerated after 14 days and re-plated in triplicates in the absence of treatment compounds (i). Sterile DMSO and a control mAb specific for the F protein of respiratory syncytial virus (palivizumab) were used as mock treatment. **(h)** sCD27 levels in supernatants (n=3 AML patients each for A and D). **(i)** Serial re-plating experiments for AML LSCs. Data are normalized to Veh control for each plating (n=3 and 5 AML patients for A and D, respectively). Significance for panels **h and i** was determined using One-way-ANOVAs followed by Tukey's multiple comparison test. **(j-o)** $5x10^6$ FACS-purified $CD45^{dim}SSC^{lo}$ cells from BM of newly diagnosed AML patients (patient P10 and P25, Supplementary Table 1) were injected intravenously into the tail vein of sub-lethally irradiated (2.75 cGy) NSG mice. After engraftment (day 32 (P10) and day 97 (P25) after transplantation), mice were randomized to treatment with control mAb and 10mg/kg αCD70 mAb (41D12-D) intraperitoneally (total of 3 injections) or decitabine and (1.5mg/kg/day) for five consecutive days[27] alone or in combination. (P10, n=2 mice/group and P25, n=4 mice/group). One day after the last treatment, animals were sacrificed and blood, spleen and BM were analyzed. **(j)** CD70 expression on $huCD45^+lin^-CD34^+CD38^-$ LSCs. Decitabine treatment is depicted in blue and vehicle treatment in black. Solid lines represent CD70 staining and dashed lines isotype control stainings on LSCs. ΔMFI: MFI staining - MFI isotype. Significance was determined using a two-sided Student's t-test for P25 ($P = 0.0015$). **(k)** sCD27 levels in sera of AML PDX mice. Statistics for P25 was determined using One-way-ANOVAs followed by Tukey's multiple comparison test. **(l)** Absolute numbers of $huCD45^+lin^-CD34^+$AML cells in the BM. Significance for P25 was determined using One-way-ANOVAs followed by Tukey's multiple comparison test. **(m)** Frequency of $CD38^-$ AML LSCs within $huCD45^+lin^-CD34^+$ AML cells. Significance was for P25 determined using One-way-ANOVAs followed by Tukey's multiple comparison test. **(n)** Human colonies per 10^5 plated BM cells from xenografted AML mice. Significance was for

P25 determined using One-way-ANOVAs followed by Tukey's multiple comparison test. **(o)** LSC frequencies estimated using ELDA. Whole BM cells at different dilutions (10^6, $5x10^5$ and 10^5, Supplementary Table 1) from treated primary AML xenograft mice were transplanted into sub-lethally irradiated (2.75 cGy) secondary recipients. Engraftment frequencies of human AML cells in mouse BM was assessed 67 (P10) and 82 (P25) later. A frequency of >0.1% of human cells (huCD45$^+$CD33$^+$) in the murine BM was rated as positive engraftment[1,28]. LSC frequencies were estimated with ELDA software (http://bioinf.wehi.edu.au/software/elda/) (see Supplementary Table 2) and significant differences in LSC frequency were calculated by $\chi 2$ (for P25: Veh vs αCD70/D: P = 1.23 e-05; D vs. αCD70/D: P = 0.0037; αCD70 vs. CD70/D: P = 0.0167). Data are represented as mean. Only statistically significant differences are indicated.

Fig. 2: The ADCC-enhanced αCD70 mAb cusatuzumab has superior efficacy in reducing LSCs than αCD70 blockade alone. (a) Lin$^-$CD90$^-$CD34$^+$CD38$^-$ LSCs (n=5 AML patients; P5, P11, P26-P27) and **(b)** Lin$^-$CD90$^+$CD34$^+$CD38$^-$ HSCs from normal control BM (n=2 controls; ctrl.4, ctrl.5) were cultured in the presence of different αCD70 mAbs (αCD70=clone 41D12-D and cusatuzumab) alone or in combination with NK cells (derived from buffy coats of healthy donors) at a ratio of 1:1 in technical triplicates overnight followed by plating in methylcellulose. Colony formation was assessed after 14 days. Fold change in colony formation is indicated vs. Veh. Statistics was determined using One-way-ANOVAs followed by Tukey's multiple comparison tests **(c-f)** $5x10^6$ FACS-purified CD45^{dim}SSC^{lo} cells from BM of patient P27 (Supplementary Table 1, Supplementary Fig. 3) were injected intravenously into sub-lethally irradiated (2.75 cGy) NSG mice. After engraftment (day 43 after transplantation), mice were randomized to treatment with control mAb, 10mg/kg αCD70 mAb (41D12-D) or cusatuzumab intraperitoneally (total of 3 injections) alone or in

combination with $1.5x10^6$ MACS-purified allogenic NK cells derived from buffy coats of heathy donors (1 injection at day 43; Groups: Veh, NK cells, αCD70, αCD70/NK cells and cusatuzumab: n=3 mice/group; Group: cusatuzumab/NK cells: n=4 mice). One day after the last treatment, animals were sacrificed, and spleen and BM were analyzed. **(d)** Frequency of human AML cells ($CD45^+CD33^+$) in the BM. Statistics was determined using One-way-ANOVA followed by Tukey's multiple comparison test. **(e)** Absolute numbers of $huCD45^+CD34^+CD38^-$ LSCs in the BM. Statistics was determined using One-way-ANOVA followed by Tukey's multiple comparison test. **(f)** Colony formation of human AML cells. 10^5 BM cells from xenografted AML mice were plated in technical duplicates and AML colonies were enumerated 14 days later. Statistics was determined using One-way-ANOVA followed by Tukey's multiple comparison test. **(g)** $Lin^-CD90^-CD34^+CD38^-$ LSCs (n=2 AML patients; P11 and P26) were cultured in the presence of NK cells at a ratio of 1:1 and cusatuzumab alone or in combination with decitabine in technical triplicates overnight followed by plating in methylcellulose. Colony formation was assessed after 14 days. Fold change in colony formation is indicated vs. NK cells. **(h)** $Lin^-CD90^-CD34^+CD38^+$ blasts and $lin^-CD90^-CD34^+CD38^-$ LSCs (n=4 AML patients; P10, P25, P32 and P33) were cultured with decitabine 0.5µM alone or in combination with cusatuzumab (10µg/ml) in the presence and absence of NK cells at a ratio of 1:1 in technical triplicates. Cell numbers were assessed after 3 days. Fold change in cell numbers per well is indicated vs. vehicle-treated cells. Statistics was determined using One-way-ANOVA followed by Tukey's multiple comparison test. **(i, j)** $lin^-CD90^-CD34^+CD38^-$ LSCs (n=5 AML patients; P8, P10, P25, P32 and P33) were cultured in the presence and absence of cusatuzumab (10µg/ml) and NK cells (ratio 1:1) in triplicates. mRNA expression of genes related to differentiation were analyzed by qRT-PCR after 48h and by Annexin V FACS staining after 72h, respectively. **(i)** Heatmap of differentiation-related genes. **(j)** Fold-change in apoptosis in LSCs (n=5 AML patients; P8, P10, P25, P32

and P33). Significance was determined using a two-sided one-sample t-test (hypothetical value =1). Data are shown as mean±S.D. Only statistically significant differences are depicted.

Fig. 3: Treatment schedule and response. (a) Treatment schedule for untreated AML patients treated with different concentrations of cusatuzumab and azacitidine. 1, 3, 10, 20 mg/kg cusatuzumab (Cusa) was given i.v. every second week. Azacitidine was administered s.c at a dose of 75 mg/kg m^2 for 7 consecutive days s.c every 4 weeks. **(b)** SWIMMR plot illustrating response and outcome of AML patients treated with cusatuzumab in combination with azacitidine. Adv., adverse risk; AE, adverse event; BR, best hematologic response; CR, complete remission; CRi, complete remission with incomplete hematologic recovery; EOT, end of treatment; fav., favorable risk; Int., intermediate risk; PR, partial remission; SD, stable disease. **(c)** Pie chart summarizing the treatment responses. CR, complete remission; CRi, complete remission with incomplete BM recovery; PR, partial remission. **(d)** Pie chart summarizing the MRD assessments in BM of CR/CRi patients**.** MRD status is depicted for only 9 out of 10 patients in CR/CRi. BM of patient C9 could not be assessed for MRD status due to short follow-up (Supplementary Table 8). **(e)** Frequency of BM blasts as determined by cytomorphology at day -14, C1D1 and BR (n=12 patients). BM blasts for patient C12 at BR are not depicted as assessment was not possible due to hypocellular BM. Statistics was determined using a two-sided paired t-test. **(f)** Representative cytomorphology for patient C2 at day -14, C1D1 and BR. Cytomorphology of all samples was assessed once. Scale bar = 10 µm. Data are represented as mean±S.D. Only statistically significant differences are depicted.

Fig. 4: Cusatuzumab reduces LSCs in AML patients. (a) CD70 expression on $CD34^-$ AML cells and $CD34^+$ AML LSPCs in the BM of patients from the study cohort and (P, n=8 patients) at day -14 (diagnosis). ΔMFI: MFI staining - MFI isotype. Sufficient material from 8

561 out 12 patients enrolled in the study was available to run the analysis. **(b)** Serum sCD27
562 (sCD27) levels from AML patients at day -14 (AML, n=12 patients) and in sera of aged-
563 matched healthy donors (HD, n=5). Detection limit (DL) of the assay: 0.2 U/ml. Measurement
564 was performed in technical duplicates. Significance was determined using a two-sided t-test.
565 **(c)** Serum sCD27 levels at day -14, C1D1 and BR (n=12 patients per time point). Dotted line
566 indicates mean sCD27 levels of healthy aged-matched control. Measurement was performed
567 in duplicates. Significance was determined using a repeated measures One-way ANOVA
568 followed by a Tukey's multiple comparisons test. **(d)** Serum sCD27 levels from AML patient
569 C2 of the study cohort at day -14, at C1D1 and BR measured in technical duplicates. **(e)** Fold
570 change in colony formation for selected patients of the study cohort (n=7). Sufficient material
571 from 7 out 12 patients enrolled in the study was available to run the analysis. Colony assays
572 were performed in technical triplicates. Significance was determined using a two-sided paired
573 t-test. **(f)** Colony formation at limiting dilution for patient C2 at day-14 and C1D1 in
574 technical triplicates. **(g)** LSC frequencies for patient C2 estimated using ELDA. Colony
575 assays were performed in technical triplicates (n=1 AML patient, C2). A frequency of >0.1%
576 of human cells (huCD45^{+}CD33^{+}) in the murine BM was rated as positive engraftment[1,28].
577 LSC frequencies were estimated with ELDA software
578 (http://bioinf.wehi.edu.au/software/elda/). **(h)** LSC frequencies for all patients with sufficient
579 cells in BM aspirates in technical triplicates (n=7 AML patients). Only sufficient material
580 from 7 out 12 patients enrolled in the study was available to run the analysis. Significance was
581 determined using a two-sided paired t-test. **(i-k)** T cell-depleted FACS-purified BM cells
582 from patient C2 at day-14 and C1D1 were injected at titrated numbers (10^6, $2{,}5x10^5$, 10^5 and
583 10^4) into sub-lethally irradiated (2.75 Gy) NSG mice (n=2 mice/ cell number injected).
584 Engraftment was assessed after 16 weeks in the BM by FACS. Detection limit for positive
585 engraftment was set as 0.1% human BM cells (CD45^{+}CD33^{+})**. (i)** Representative FACS plots
586 of engraftment of human cells in the BM of PDX mice from patient C2. Data from one single

experiment are shown. **(j)** LSC frequencies estimated using ELDA. A frequency of >0.1% of human cells (huCD45$^+$CD33$^+$) in the murine BM was rated as positive engraftment[1,28]. LSC frequencies were estimated with ELDA software (http://bioinf.wehi.edu.au/software/elda/). **(k)** Fold change in stem cell frequency. **(l)** Heatmap. Common differentially regulated genes after cusatuzumab treatment in LSPCs of patients C8 and C10 identified via scRNA sequencing. **(m)** Histogram of GO enrichment analysis of the biological pathways significantly affected in Lin$^-$CD34$^+$ AML cells after cusatuzumab monotherapy. **(n)** Fold-change in apoptosis in lin$^-$CD90$^-$CD34$^+$ LSPCs in BM of AML patients at C1D1 versus day-14 as analyzed by Annexin V FACS staining (n=7 patients). Significance was determined using a two-sided one-sample t-test (hypothetical value =1). Data are represented as mean. Only statistically significant differences are indicated.

Online methods

Detailed information on experimental design and reagents can be found in the Life Sciences Reporting Summary.

Animals. NOD/LtSz-scid IL2Rgnull (NSG) mice were purchased from Charles River (Sulzfeld, Germany). 6-8 weeks old male and female mice were housed under specific pathogen-free conditions in individually ventilated cages with food and water ad libitum and were regularly monitored for pathogens. All animals used in the experiments were age- and sex-matched Randomization of mice into treatment group was performed using GraphPad software random number generator. Experiments were conducted and analyzed in a non-blinded fashion. Details on repetitions and replicates are indicated in the Fig. legends. Experiments were approved by the local experimental animal committee of the Canton of Bern and performed according to Swiss laws for animal protection (BE75/17 and BE78/17).

Cell line. MOLM-13 and NOMO-1 cells were specifically purchased from ATCC for this study. Therefore, the authors performed no additional authentication. The cell line was tested mycoplasma-free and was grown in FCS-containing medium recommended by ATCC (https://www.lgcstandards-atcc.org/?geo_country=ch) with GlutaMAX supplemented 100 U/mL penicillin, and 100 µg/mL of streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Patients samples for preclinical experiments. Peripheral blood samples and BM aspirates were obtained from untreated AML patients at diagnosis and HMA-treated AML patients at the University Hospital of Bern, Switzerland between 2014-2017 (Supplementary Table 1). Informed written consent was collected from all patients involved in the study. Study data were collected and managed using REDCap electronic data capture tools hosted at the Department for BioMedical Research[29]. Diagnostic BM aspirates that were considered normal according to hematologist and a surgical pathologist were used as controls.
Serum samples from elderly healthy donors (n=5; 69.2 ± 1.8 yrs) and buffy coats from healthy donors were obtained from the regional blood transfusion service in Bern. Analysis of samples was approved by the local ethical committee of Bern, Switzerland (KEK 122/14).

Antibodies, flow cytometry and reagents for treatment. αCD34-APC (cat. 343607, clone 561, 1:80), αCD34-PE (cat. 343603, clone 561, 1:100), αCD38-PE-Cy7 (cat. 303515, clone HIT2, 1:50), αCD90-PerCP-Cy5.5 (cat. 382113, clone 5E10, 1:100), αCD33-PerCP-Cy5.5 (cat 302413, clone WM53, 1:100), CD45RA-APC-Cy7 (cat. 304151, clone HI100, 1:20), αCD8a-PerCP-Cy5.5 (cat. 300923, clone HIT8a, 1:100), αCD4-PerCP-Cy5.5 (cat. 344607, clone SK3, 1:100), CD19-APC-Cy7 (cat 302217, clone HIB19, 1:100), mouse-αCD45-

PerCP-Cy5.5 (cat. 103131, clone 30-F11, 1:200), mouse-αCD45-PE-Cy7 (cat. 103113, clone 30-F11, 1:200), Annexin-V-Pacific blue (1:50) were from BioLegend. Lineage-positive cells were excluded by staining using biotinylated αCD2 (cat. 300203, clone RPA2.10, 1:100), αCD3 (cat. 317319, clone OKT3, 1:100), aCD14 (cat. 325623, clone HCD14, 1:100), αCD16 (cat. 302003, clone 3G8, 1:100), αCD19 (cat. 302203, clone HIB19, 1:100), αCD56 (cat. 318319, clone HCD56, 1:100) and αCD235 (cat. 306617, clone HIR2, 1:100) (BioLegend), followed by a second step using streptavidin-FITC (BD Pharmingen, 1:3000). αCD70-PE (cat. 555835, clone KI-24, 1:10) and corresponding isotype control mAb were from BD Pharmingen. Viability dye eFluor450 was from eBiosciences (1:1000). αCD45-V-500C (cat. 56077; clone 2DI, 1:50) was from BD Pharmingen.

Flow cytometric analysis on whole BM was performed following red blood cell (RBC) lysis. Samples were analyzed on a BD Fortessa and sorting procedures were performed using a BD FACS Aria III (BD Pharmingen). Data were collected using FACSDiva software (BD Pharmingen) analyzed using FlowJo software (Treestar). Effective separation after FACS-sorting was assessed by re-analyzing a fraction of the sorted samples by flow-cytometry analysis (purity after FACS-sorting: 95.3 ± 1.3%). Human αCD70 mAb (41D12-D) specifically blocks the CD70/CD27-interaction with deficiency in effector functions by E233P/L234V/L235A amino acid substitutions in the CH2 domain of the parental clone 41D12-D[18,19], cusatuzumab and pavilizumab (Synagis®, mock treatment) were from argenx.

RNA isolation and qRT-PCR. For qRT-PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen). Complementary DNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression analysis was performed using self-designed primers for *CD70* (FW, TGCTTTGGTCCCATTGGTC; RV, TACGTCCCACCCAAGTGAC), *CEBPA* (FW, AGACCTAGAGATCTGGCTGTG; RV,

GGACTGATCGTGCTTCGTG), *CEBPB* (FW, TTTCGAAGTTGATGCAATCGG; RV, AAACATCAACAGCAACAAGCC), *ID1* (FW: TGTTACTCACGCCTCAAGGA; RV: CTGAAGGTCCCTGATGTAGTC), *RUNX1* (FW: GCTTCACTCTGACCATCACTG; RV: TGCCGATGTCTTCGAGGT), SPI1 (FW: CCTCAGCCATCAGAAGACCT; RV: CAGTAATGGTCGCTATGGCTC), *SP-1* (FW, AGAGGCCATTTATGTGTACCTG; RV, AGGGCAGGCAAATTTCTTCTC), and *GAPDH* (FW, TCATTTCCTGGTATGACAACGA; RV, CTTCCTCTTGTGCTCTTGCTG) using SYBR Green Reaction. qRT-PCR reactions were performed in duplicates including non-template controls using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Relative quantification of gene expression was normalized against a reference gene (*GAPDH*) and calculated as an exponent of 2 ($2^{\Delta Ct}$).

MicroRNA analysis. Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and cDNA synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). MicroRNA concentrations were measured using TaqMan miRNA assays for miR-29b, or RNU48 (Applied Biosystems), and qRT-PCR results were normalized to RNU48 expression.

Liquid cultures. $1x10^5$ FACS-purified $CD34^+CD38^-$ LSCs from BM of AML patients and or HSCs from normal control BM (ctrl., Supplementary Table 1) were cultured in StemSpan SFEM medium (Stem Cell Technologies) supplemented with human cytokines (StemSpan CC100; Stem Cell Technologies) in the presence or absence of 10 mg/ml αCD70 mAb or 0.5 µM decitabine or azacitidine alone or in combination in 96-well plates at 37°C and 5% CO_2. Numbers of viable cells were assessed by trypan blue staining or viability dye staining. CD70 mRNA and protein expression and sCD27 levels were determined after 3 days of culture.

688 **Colony assays.** Colony assays of FACS-purified $CD34^+CD38^-$ LSCs from the peripheral
689 blood or BM of newly diagnosed AML patients or normal control BM (ctrl., Supplementary
690 Table 1) were performed as previously described with slight modifications[30]. Briefly, 1×10^3
691 $CD34^+CD38^-$ cells were cultured overnight in 96-well plates at 37°C and 5% CO_2 in
692 StemSpan SFEM medium in the presence or absence of 10 mg/ml αCD70 mAb or 0.5 µM
693 decitabine or azacitidine alone or in combination followed by plating into MethoCult H4435
694 enriched medium (methylcellulose, STEMCELL Technologies). αCD70 and HMA were
695 added in the first cultures but not during re-plating. Sterile DMSO and a control mAb specific
696 for the F protein of respiratory syncytial virus (palivizumab) were used as mock treatment.
697 Colonies and cells were enumerated after 14 days (≥30 cells/colony). For serial re-plating
698 experiments, 10^4 cells were collected from preceding colony assays and were re-plated in
699 methylcellulose without further addition of mAb or HMA. Colonies were enumerated 14 days
700 later.
701
702 **Murine patient-derived xenograft AML model and limiting dilution experiments.**
703 Patient-derived xenotransplantation experiments were performed as previously described[11].
704 NSG mice were sub-lethally irradiated (2.75 Gy) on the day before injection. 5×10^6 FACS-
705 purified $CD45^{dim}SSC^{lo}$ from BM of newly diagnosed AML patients (patient P10, P25 and
706 P27, Supplementary Table 1) were injected intravenously into the tail vein. The $CD34^+$ cell
707 frequencies for PDX of P10: 90.4±0.2, for PDX 25: 5.8±0.8, and for PDX 27: 81,8±1.0.
708 Five weeks (P10), 12 (P25) and 6 weeks (P27) after transplantation, mice were randomized
709 using GraphPad software random number generator into the different treatment groups as
710 specified in the respective figure legends.
711 For the analysis of BM in xenografted AML mice, the immunophenotype of LSCs was
712 defined as $lin^-CD34^+CD38^-$ cells according to [1,31,32].

For limiting dilution assays, whole BM cells from treated primary AML xenograft mice or AML study patients at diagnosis and after cusatuzumab monotherapy were transplanted at titrated numbers into sub-lethally irradiated (2.75 Gy) secondary recipients and engraftment in blood, spleen and BM was assessed at time points indicated in figure legends. A frequency of >0.1% of human cells (huCD45$^+$CD33$^+$) in the murine BM was rated as positive engraftment. To study LSC frequencies for patient C2 at day-14 and C1D1 in vivo, FACS-purified T-cell depleted BM cells were injected at titrated numbers into sub-lethally irradiated (2.75 Gy) secondary recipients. Engraftment was assessed after 16 weeks in the BM by FACS. A frequency of >0.1% of human cells (huCD45$^+$CD33$^+$) in the murine BM was rated as positive engraftment.

sCD27 measurements

sCD27 levels in cell supernatants and in serum of PDX mice were analyzed by ELISA as previously described in [11]. Soluble CD27 (sCD27) in serum from patients enrolled in the clinical trial was measured in duplicates using the CD27 (Soluble) Human Instant ELISA™ Kit (Invitrogen). Detection limit of the assay was 0.2 U/ml.

DNA methylation analysis of the *CD70* promoter. The methylation of the SP-1 binding site at the CD70 promoter was analyzed in FACS-purified lin$^-$CD90$^-$CD34$^+$CD38$^-$ AML LSCs and lin$^-$CD90$^+$CD34$^+$CD38$^-$ HSCs from newly diagnosed AML patients and healthy donors (HD) as well as from lin$^-$CD90$^-$CD34$^+$ AML cells which were treated with vehicle or decitabine at 0.5µM overnight in 3 independent replicates per condition.

Bisulfite conversion of isolated DNA of cells was assessed using the Epitect® Bisulfite Kit (Qiagen) according to the manufacturer's protocol. The promoter region covering binding sites for important transcription factors was selectively amplified using the following primers:

738 forward primer 5'- GAGAGGGGTATACGAATATTTGG-3'; reverse primer 5'-
739 ACCGCTACCAATCTAAAAATCC -3'. For the amplification of bisulfite-treated genomic
740 DNA (gDNA), the following PCR conditions were used: 1x 95°C for 10min; 40x 95°C for
741 30s, 56°C for 30s, 72°C for 1min; 1x 72°C for 5min. The PCR cocktail consisted of 3µl of
742 DNA (of at least 10ng/µl DNA for a final concentration of 3ng/µl per reaction) in a 25µl total
743 volume using 1µl of each primer (10µM), 200µM dNTPs, 0.2U hot start Taq DNA
744 polymerase, 2µl Q-solution 5x (Qiagen), 1.5mM $MgCl_2$ and the buffer supplied with the
745 enzyme. Subsequent nested PCR was performed with the following primers: forward primer
746 5' -GAGTATTTTTAATTTTTGGATGTTTGTTG-3' and reverse primer 5'-
747 ACAATTACCAAAATACAAACAATAACC-3', using the same PCR conditions as
748 described above for bisulfite sequencing. The amplified promoter region was gel-purified and
749 subjected to fluorescent Sanger sequencing. The relative quantification of the methylated
750 allele (C) versus the unmethylated allele (T) was assessed using the QSVAnalyser software
751 (University of Leeds)[33].
752
753 **Determination of synergy.** Synergy between compounds was quantified using the Chou-
754 Talalay method[22]. For synergy studies, we calculated the % reduction of cell numbers after
755 drug treatments compared to vehicle-treated cells. We determined the half-maximal inhibitory
756 concentrations (IC_{50}) of cusatuzumab and decitabine in the presence of NK cells (derived
757 from buffy coats of healthy donors) and treated MOLM-13 cells with titrated concentrations
758 of the compounds, below, or above the IC_{50}. Dose effect analysis, the dose response values
759 IC_{50}, the slope (m) and the correlation coefficient (r) for the single treatments, as well as the
760 combination index (CI) that reflects the extent of synergy or antagonism for two drugs were
761 determined using CompuSyn software. CI<1, synergy; CI=1, additivity; CI>1, antagonism.
762

Single-cell RNA sequencing (scRNA-seq) workflow and analysis

Barcoded cDNA from of at least 977 $lin^{-}CD34^{+}$ cells from patients C8 and C10 was prepared using the Chromium Single Cell 3′ Library (v2 Chemistry) & Gel Bead Kit on a Chromium Controller according to the manufacturer's recommendations (10XGenomics). The barcoded cDNA was further processed into scRNA-seq libraries and sequenced on a NovaSeq 6000 instrument using NovaSeq Control Software v1.6 (Illumina). Libraries were sequenced with 100 cycle S1 flow cell kits using the following paired-end configuration: 26 bp Read 1, 91 bp Read 2.

For post-processing of the samples and alignment of the cDNA reads to the human reference transcriptome (GRCh38), the CellRanger package (v2.1.1, 10X Genomics) was employed[34]. This process allocates a unique molecular identifier (UMI, transcript) expression matrix to only those single cells containing a cell-barcode (filtered feature-barcode matrix). The expression matrix of each single cell was then normalized by the "cellranger aggr" function from the CellRanger package. This resulted in the identification of 4,883 cell-barcodes with a median UMI per cell of 9972 and median expression of 2400 genes per cell for patient C8 and 2266 cell-barcodes with a median UMI per cell of 9138 and median expression of 2215 genes per cell for patient C10. Subsequently, the Seurat package (v2.3.4) was used for quality control (filtering), graph-based clustering, visualization and differential gene expression analysis for on single cell level[35]. Single cells identified by the Seurat package as outliers (>3500 expressed genes) and cells that showed a high level (>5%) of reads that aligned to the mitochondrial genome were removed[36]. To discriminate between malignant and healthy single cells in our samples, we took advantage of the molecular mutations identified at diagnosis in patients C8 and C10 and performed a data crushing approach. Single cells with a log2 gene expression of $\geq 10^{-5}$ and $<10^{-5}$ of the respective marker(s) were considered as healthy cells and malignant cells, respectively. Unsupervised cell-clustering and differential genes expression analysis of malignant cells for patients C8 and C10 at day -14 and C1D1 was performed using

the Seurat package and the Seurat "findMarker" function, respectively. All figures and tables related to the scRNA-seq analysis were generated based on the filtered malignant cells.

Patients and study design. Argx-110-1601 was a phase 1/2, open-label, dose-escalating study (n=12 patients) with a proof of concept cohort (n=26 patients) to evaluate the safety, tolerability, and efficacy of ARGX-110 in combination with azacytidine in subjects with newly diagnosed acute myeloid leukemia (AML) or high-risk myelodysplastic syndrome (MDS) (NCT03030612). This manuscript reports the results of the dose-escalation part of the phase 1/2 study (n=12 patients). The dose-escalation part used a 3 + 3 design to establish the maximum-tolerated dose of cusatuzumab in combination with azacitidine. Cusatuzumab was administered intravenously every 2 weeks (first dose at day -14, all further applications on days 3 and 17 of all therapy cycles) at 1, 3, 10 and 20 mg/kg b.w. in combination with standard doses of subcutaneous azacitidine of 75 mg/m^2 given at days 1 to 7 every 28 days. Main inclusion criteria were: untreated AML ≥ 20% blasts, age ≥ 18 years, expected life expectancy ≥ 3 months, ECOG performance status of 0, 1, or 2. Main exclusion criteria were: Prior or concurrent malignancy, any previous cancer chemo- or radiotherapy, abnormal organ function (aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) > 3 x upper limit of normal (ULN) or in case of liver infiltration by AML, AST and/or ALT > 5 x ULN; Alkaline phosphatase (AP) > 2.5 x ULN or in case of liver infiltration by AML, AP > 5 x ULN; Serum (total) bilirubin > 1.5 x ULN or in case of liver infiltration by AML, serum (total) bilirubin > 5 x ULN; Serum creatinine > 2.5 x ULN or GFR (MDRD) of < 40 mL/min for patients with creatinine levels above the normal limit); use of immune-suppressive agents for the past 4 weeks. The primary objective of the phase 1 was to determine the MTD of cusatuzumab (formerly ARGX-110) and/or the recommended phase 2 dose (RP2D) in combination with a standard dose of azacitidine. The secondary objectives involved evaluating the safety and tolerability, the pharmacokinetics and immunogenicity, the

pharmacodynamics and preliminary efficacy of multiple ascending intravenous doses of cusatuzumab in combination with standard doses of azacitidine.

The trial (EudraCT number 2016-002151-17) was sponsored by argenx, (Belgium) and conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practices Guidelines. The clinical study protocol and its amendments, informed consent documents, and any other appropriate study-related documents were reviewed and approved by the applicable ethics committee of Bern, Switzerland, and the national regulatory authorities Swissmedic in Bern, Switzerland. Patients in the phase 1 part of the trial were enrolled between January 2017 and March 2018 at the Inselspital, Bern University Hospital and the University Hospital Zürich.

Samples were collected at diagnosis (day -14) and for response assessments of cusatuzumab monotherapy (C1D1) and at regular intervals during the trial. Pharmacodynamics as well as pharmacokinetic samples were collected during monotherapy as well as combination therapy. The intermediate cut-off for this report in the ongoing study was February 18th 2019.

Response criteria

The hematologic remission status was assessed according to the criteria of the International Working Group[15,37]. MRD was evaluated by multi-parameter flow cytometry according to the criteria of the European Leukemia Net (ELN). The detection of at least 0.1% ($>10^{-3}$) of cells with a leukemia-associated immunophenotype was considered as positive MRD result[38].

Cytogenetic and mutation analysis

Chromosome banding analysis from bone marrow was done according to standard procedures with a minimum of 20 metaphases being required for a valid report. Fluorescence in situ hybridization (FISH) and/or array comparative hybridization (aCGH) were added if needed for further clarification or for confirmation of the results of chromosome banding analysis.

841 Mutations detected at diagnosis in the 12 patients in this study (e.g. *CEBPA, EZH2, RUNX1,*
842 *SH2B3, ZRSR2, NPM1*; see Supplementary Table 3) were determined by NGS.
843
844 **Statistical analysis**
845 Statistical analysis was performed using GraphPad Prism® software v7.0 (GraphPad). Bars
846 and error bars indicate means and standard deviations if not otherwise specified in Fig.
847 legends. All statistical tests were two-sided and P values ≤ 0.05 (95% confidence interval)
848 were considered statistically significant. Normality was measured using the D'Agostino &
849 Pearson normality test for all experiments with $n \geq 8$. Correlations were performed using a
850 Pearson correlation coefficient. Data were analyzed using one-sample test (hypothetical value
851 =1); Student's t-test, paired t-test, one-way ANOVA followed by Tukey's multiple
852 comparison test, repeated measure one-way ANOVA followed by Tukey's multiple
853 comparison test. The test applied to determine significances is specified in detail in the
854 corresponding figure legend. LSC frequencies with 95% confidence intervals (CI) were
855 estimated with Extreme Limiting Dilution Analysis software
856 (http://bioinf.wehi.edu.au/software/elda/) and significant differences in LSC frequency were
857 calculated by $\chi 2$ test in limiting dilution assays according to[21]. The statistical analysis of
858 differentially expressed genes in $CD34^+$ AML cells before and after cusatuzumab treatment
859 was performed using a Wilcoxon rank sum test followed by a P value adjustment using
860 Bonferroni correction.
861
862 **Data availability statement**
863 Patient-related clinical data sets in the paper were generated during and analyzed as part of the
864 part of a multicenter clinical trial (NCT03030612). The data sets are not publicly available
865 because the trial is still ongoing, but data can be requested by any qualified researcher after

data lock and completion of trial or on reasonable request.

All RNA raw data, the analyzed sequencing data can be found at the GEO database and are available via accession number GSE147989 (single cell RNA-seq).

All other data that support the findings of the study are available from the corresponding author upon request.

Code availability statement

The code used for the analysis of the single cell sequencing data are available from the corresponding author upon request.

Methods only references

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Figure 1

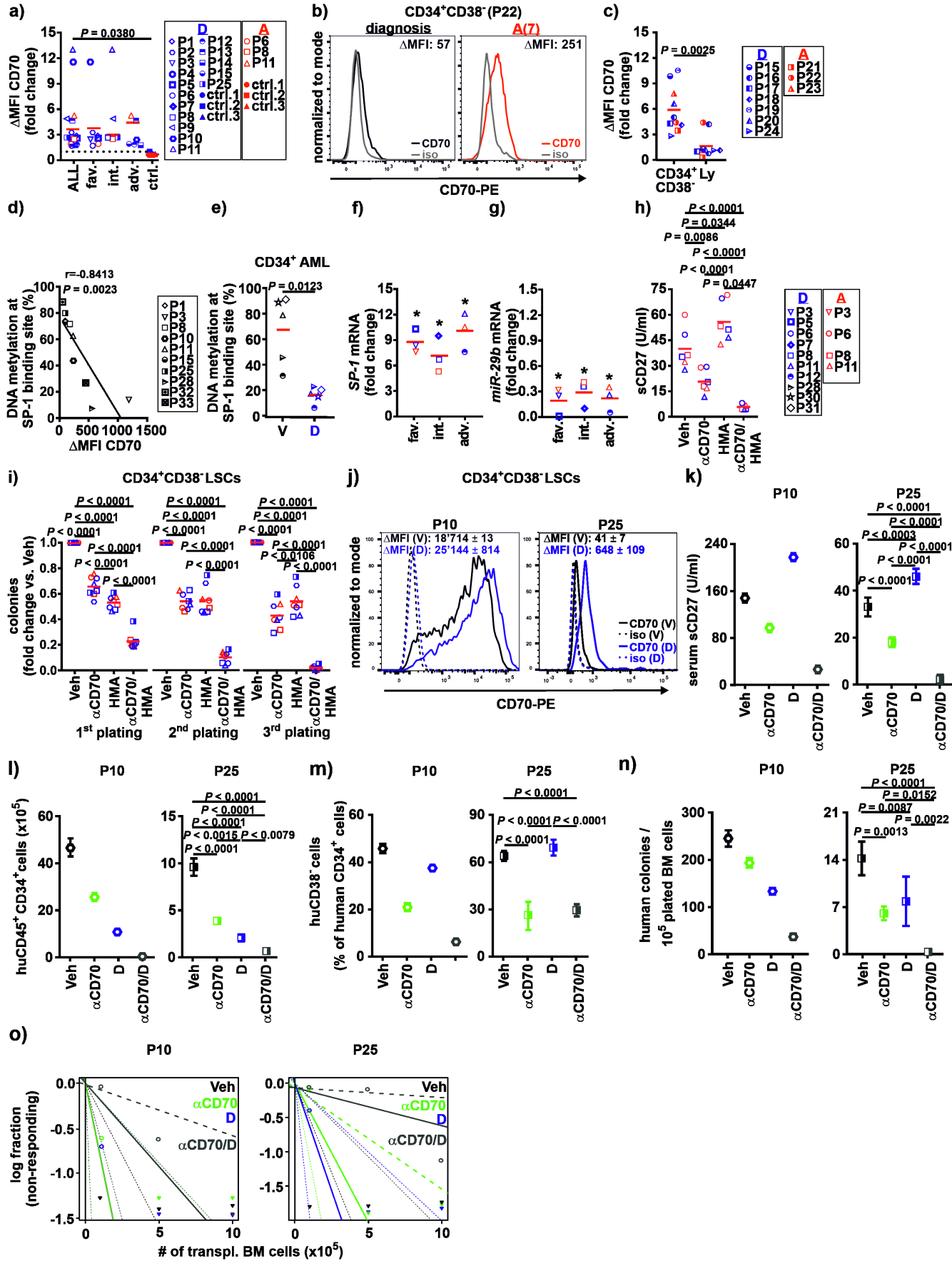

a)
ΔMFI CD70 (fold change)
P = 0.0380
ALL
fav.
int.
adv.
ctrl.
D
A
b)
CD34+CD38- (P22)
diagnosis
A(7)
ΔMFI: 57
ΔMFI: 251
normalized to mode
CD70
iso
CD70-PE
c)
P = 0.0025
CD34+ Ly CD38-
d)
r=-0.8413
P = 0.0023
DNA metylation at SP-1 binding site (%)
ΔMFI CD70
e)
CD34+ AML
P = 0.0123
V
D
f)
SP-1 mRNA (fold change)
g)
miR-29b mRNA (fold change)
h)
sCD27 (U/ml)
Veh
αCD70
HMA
αCD70/ HMA
i)
CD34+CD38- LSCs
colonies (fold change vs. Veh)
1st plating
2nd plating
3rd plating
j)
CD34+CD38- LSCs
P10
P25
ΔMFI (V): 18'714 ± 13
ΔMFI (D): 25'144 ± 814
ΔMFI (V): 41 ± 7
ΔMFI (D): 648 ± 109
CD70 (V)
iso (V)
CD70 (D)
iso (D)
CD70-PE
k)
serum sCD27 (U/ml)
αCD70/D
l)
huCD45+ CD34+ cells (x10^5)
m)
huCD38- cells (% of human CD34+ cells)
n)
human colonies / 10^5 plated BM cells
o)
log fraction (non-responding)
# of transpl. BM cells (x10^5)

Figure 2

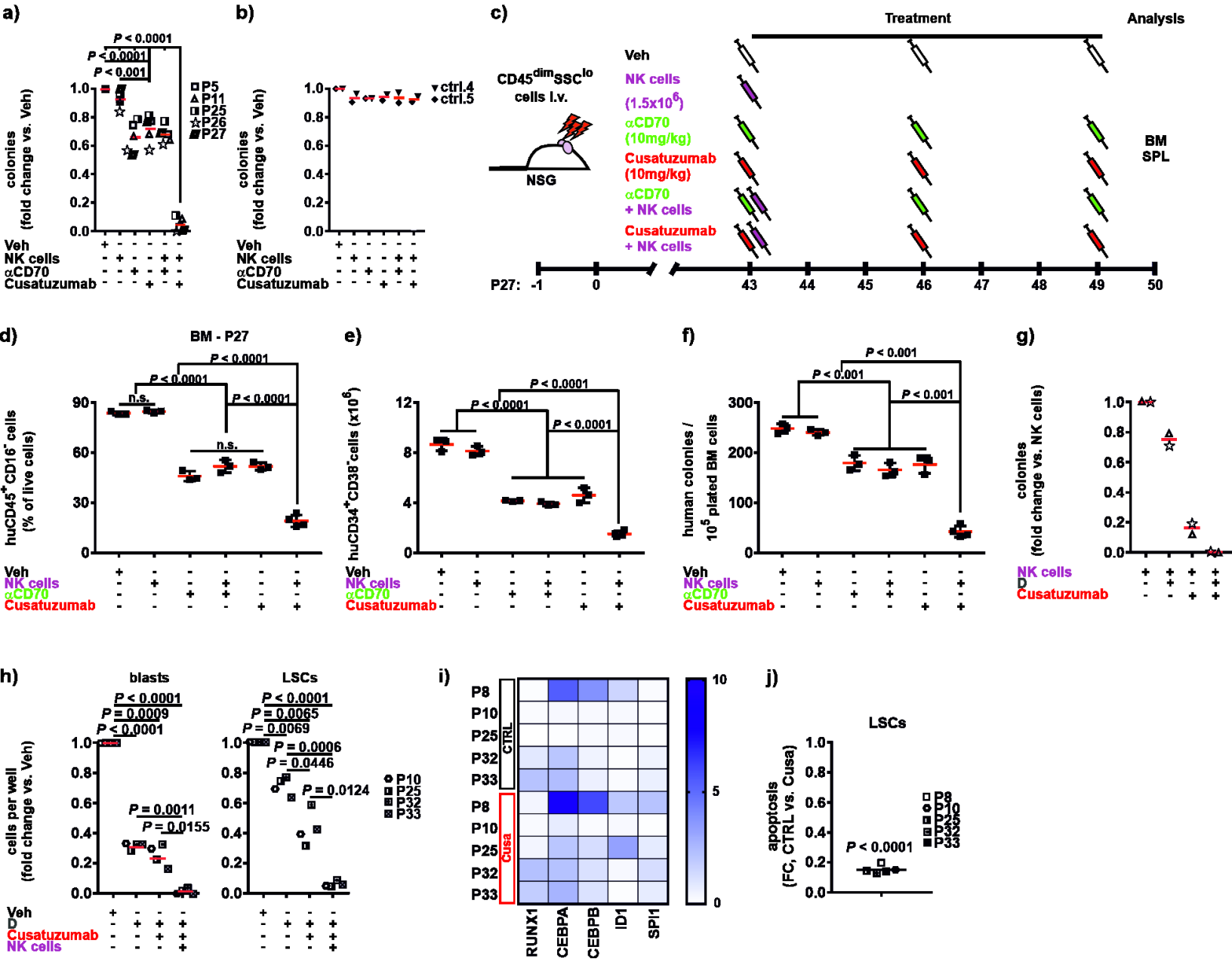
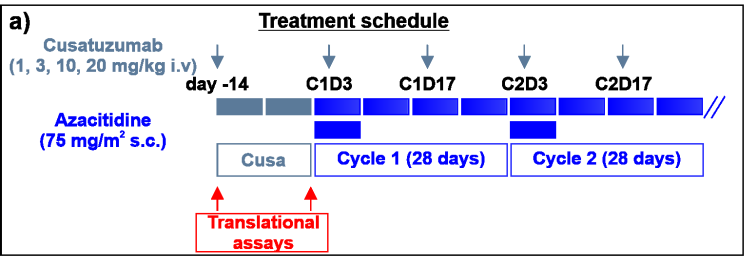
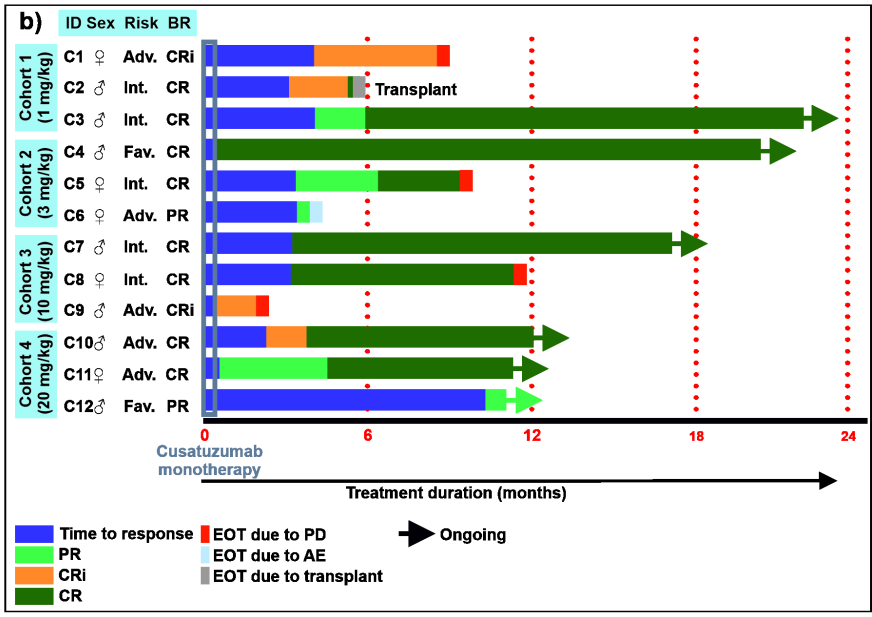
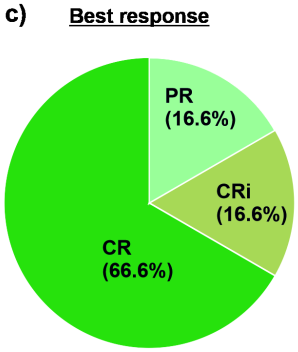
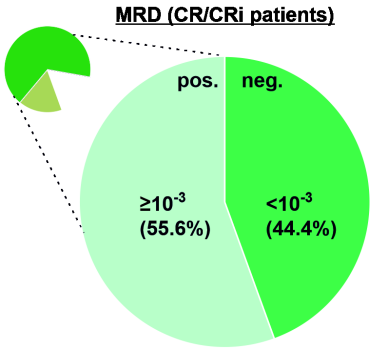

a)
b)
c)
Treatment
Analysis
Veh
NK cells
(1.5x10^6)
αCD70
(10mg/kg)
Cusatuzumab
(10mg/kg)
αCD70
+ NK cells
Cusatuzumab
+ NK cells
CD45^dim SSC^lo
cells i.v.
NSG
BM
SPL
P27: -1 0 43 44 45 46 47 48 49 50
d)
BM - P27
e)
f)
g)
h)
blasts
LSCs
i)
j)
LSCs
RUNX1
CEBPA
CEBPB
ID1
SPI1
CTRL
Cusa

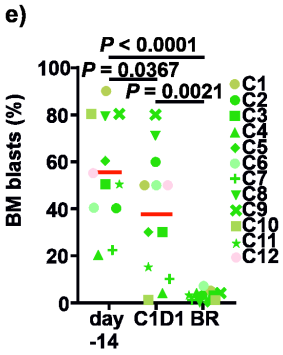
Figure 3


a)
Treatment schedule
Cusatuzumab
(1, 3, 10, 20 mg/kg i.v)
day -14
C1D3
C1D17
C2D3
C2D17
Azacitidine
(75 mg/m² s.c.)
Cusa
Cycle 1 (28 days)
Cycle 2 (28 days)
Translational assays


b)
ID Sex Risk BR
Cohort 1 (1 mg/kg)
C1 ♀ Adv. CRi
C2 ♂ Int. CR
C3 ♂ Int. CR
Transplant
Cohort 2 (3 mg/kg)
C4 ♂ Fav. CR
C5 ♀ Int. CR
C6 ♀ Adv. PR
Cohort 3 (10 mg/kg)
C7 ♂ Int. CR
C8 ♀ Int. CR
C9 ♂ Adv. CRi
Cohort 4 (20 mg/kg)
C10♂ Adv. CR
C11♀ Adv. CR
C12♂ Fav. PR
0
6
12
18
24
Cusatuzumab monotherapy
Treatment duration (months)
Time to response
PR
CRi
CR
EOT due to PD
EOT due to AE
EOT due to transplant
Ongoing


c)
Best response
PR (16.6%)
CRi (16.6%)
CR (66.6%)


d)
MRD (CR/CRi patients)
pos.
neg.
≥10-3 (55.6%)
<10-3 (44.4%)


e)
P < 0.0001
P = 0.0367
P = 0.0021
BM blasts (%)
100
80
60
40
20
0
day -14
C1D1
BR
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12

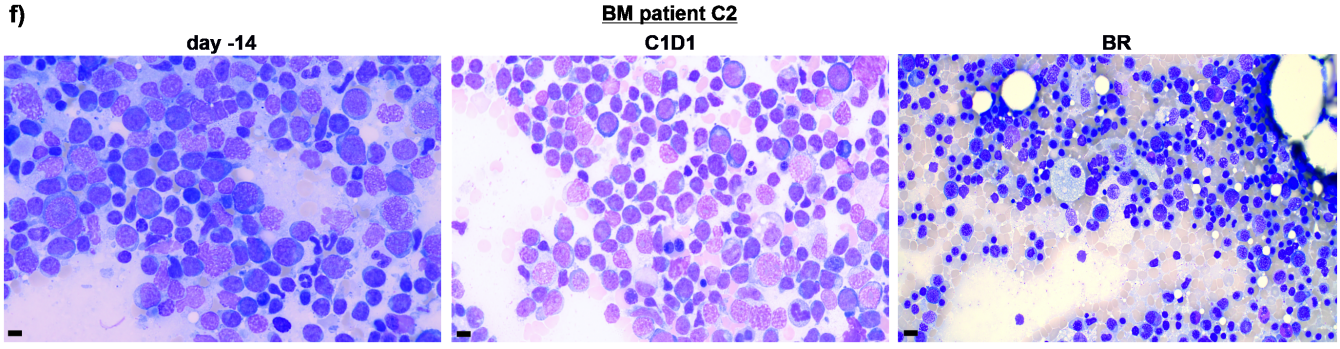
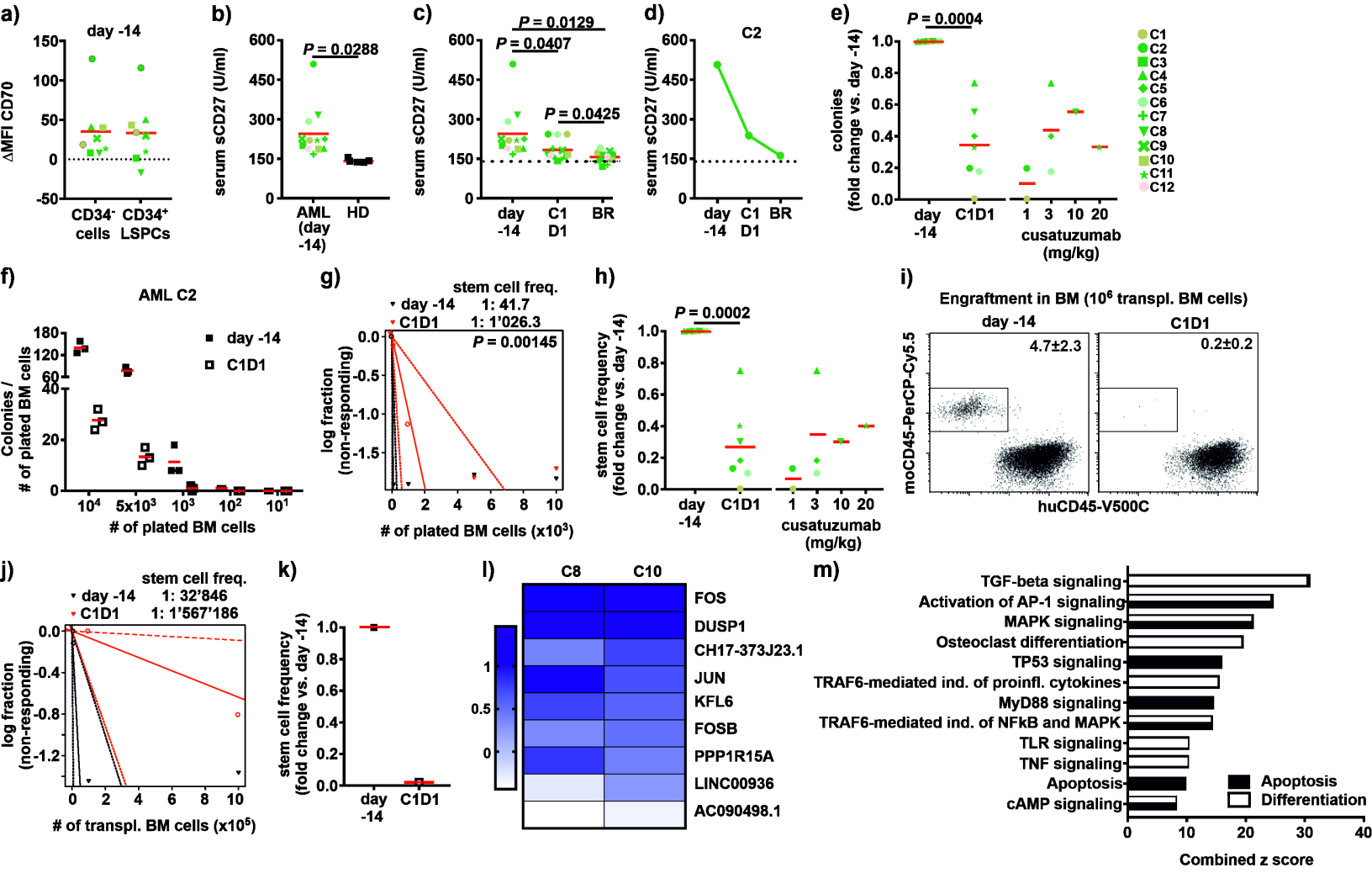

f)
BM patient C2
day -14
C1D1
BR

Figure 4


a)
day -14
ΔMFI CD70
CD34- cells
CD34+ LSPCs
b)
serum sCD27 (U/ml)
P = 0.0288
AML (day -14)
HD
c)
P = 0.0129
P = 0.0407
P = 0.0425
day -14
C1 D1
BR
d)
C2
e)
P = 0.0004
colonies (fold change vs. day -14)
day -14
C1D1
cusatuzumab (mg/kg)
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
f)
AML C2
Colonies / # of plated BM cells
day -14
C1D1
# of plated BM cells
g)
stem cell freq.
day -14 1: 41.7
C1D1 1: 1'026.3
P = 0.00145
log fraction (non-responding)
# of plated BM cells (x10³)
h)
P = 0.0002
stem cell frequency (fold change vs. day -14)
i)
Engraftment in BM (10⁶ transpl. BM cells)
day -14
C1D1
4.7±2.3
0.2±0.2
moCD45-PerCP-Cy5.5
huCD45-V500C
j)
stem cell freq.
day -14 1: 32'846
C1D1 1: 1'567'186
log fraction (non-responding)
# of transpl. BM cells (x10⁵)
k)
stem cell frequency (fold change vs. day -14)
l)
C8
C10
FOS
DUSP1
CH17-373J23.1
JUN
KFL6
FOSB
PPP1R15A
LINC00936
AC090498.1
m)
TGF-beta signaling
Activation of AP-1 signaling
MAPK signaling
Osteoclast differentiation
TP53 signaling
TRAF6-mediated ind. of proinfl. cytokines
MyD88 signaling
TRAF6-mediated ind. of NFkB and MAPK
TLR signaling
TNF signaling
Apoptosis
cAMP signaling
Apoptosis
Differentiation
Combined z score
n)
CD34+ LSPCs
P = 0.0336
apoptosis (FC, C1D1 vs. day -14)
CR
CRi
PR