SUPPLEMENTARY APPENDIX

The interleukin-3 receptor CD123 targeted SL-401 mediates potent cytotoxic activity against CD34+CD123+ cells from acute myeloid leukemia/myelodysplastic syndrome patients and healthy donors

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The interleukin-3 receptor CD123 targeted SL-401 mediates potent cytotoxic activity against $CD34^+CD123^+$ cells from acute myeloid leukemia / myelodysplastic syndrome patients and healthy donors

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Additional Supporting Information supplied in Online Supplementary File:

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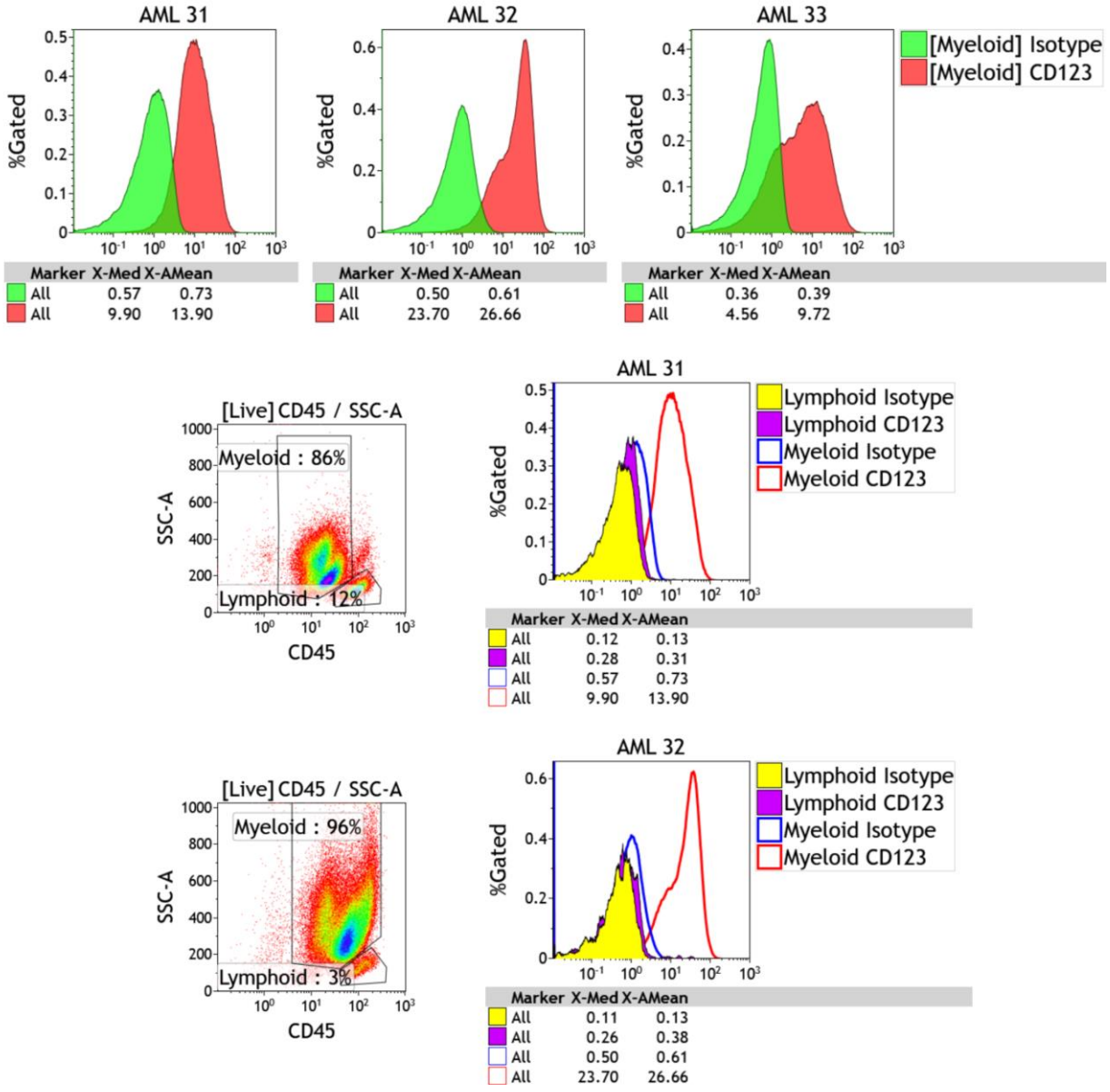
Supplementary Methods

***Chemicals and reagents*:** Antibodies (clones) used include CD34-FITC/BV421 (561), CD33-PE (WM53), CD45-PE Dazzle/FITC/APC (HI30), CD3-PE-Cy7 (SK7), CD123-APC (6H6), isotype IgG1κ-APC (MOPC-21), HLA-DR-BV510 (L243), CD19-BV785 (SJ25C1), CD105-FITC (43A3), CD73-PE (AD2), CD90-APC (5E10) (Biolegend, San Diego, CA), anti-human CD3 (OKT3) and murine CD45-PECF594 (30-F11) (BD Biosciences, San Jose, CA). Annexin V-FITC or PE, propidium iodide and 7AAD were purchased from BD Biosciences. LIVE/DEAD near-IR stain (Invitrogen) and Count Bright™ absolute counting beads (Life Technologies) were used for flow cytometry assays. CD34 MicroBead Kit Ultrapure (Miltenyi Biotec, CA, USA) was used to purify $CD34^+$ cells from cord blood samples. Human methylcellulose complete media without erythropoietin (R&D Systems) was used for colony forming cell (CFC) assays.

AML Patient-Derived Xenograft (PDX) models: Animal experiments were performed under a protocol approved by the OSU Institutional Animal Care and Use Committee (IACUC). Primary AML cells were cultured for up to 72 hours after thawing cryovials with IL-3, GM-CSF and SCF (10ng/ml). Anti-CD3 antibody (OKT3) was used to deplete T cells in the AML culture. Six-to-eight week-old NRG-SGM3 (NRGS) mice (Division of Hematology, OSU) were preconditioned with busulfan (20mg/kg, intraperitoneal administration) 24 hours prior to intravenous injection of 4-6 x 10^6 primary AML cells pre-tested for engraftment potential. Ten days after engraftment, mice were randomized and treated with vehicle or SL-401 (50µg/kg administered intraperitoneally, three doses: M/W/F per week for 5 weeks). All animals were monitored for signs of disease and other exclusion criteria including extended anorexia and weight loss.

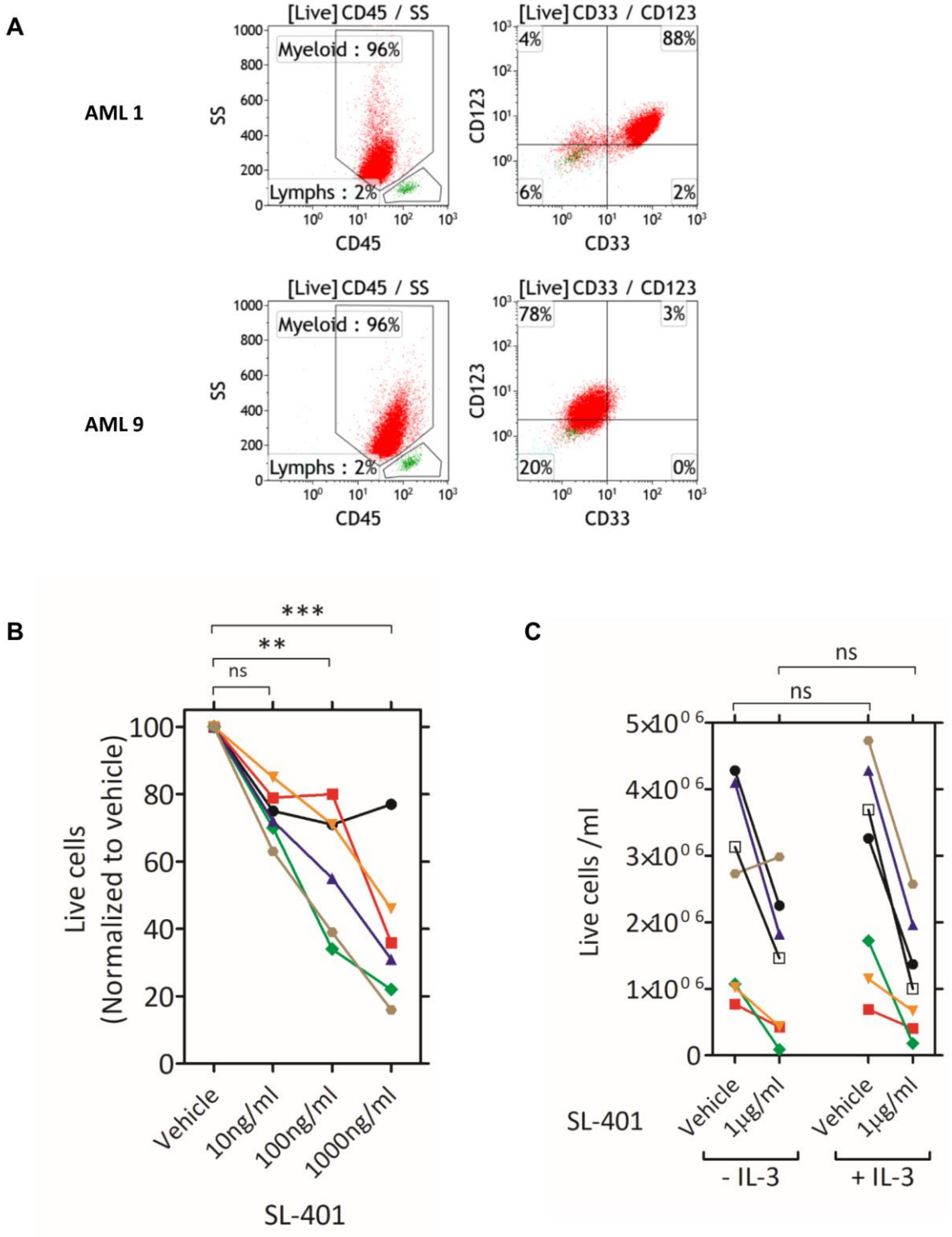
Statistics: Cytotoxicity data were analyzed by mixed effect model, incorporating repeated measures for each subject. Multiplicity was adjusted by Holm's method. Paired t-test was used for matched sample comparisons. The correlation between CD123 levels and SL-401 were assessed by Pearson correlation method and visualized by matrix plots (N=16). Two-sample tests were used to compare the means of survival days between treatment and control groups. Data analyses were performed by using SAS 9.4 (SAS, Inc.; Cary, NC). ***P<0.0001; **P<0.001; *P<0.05.

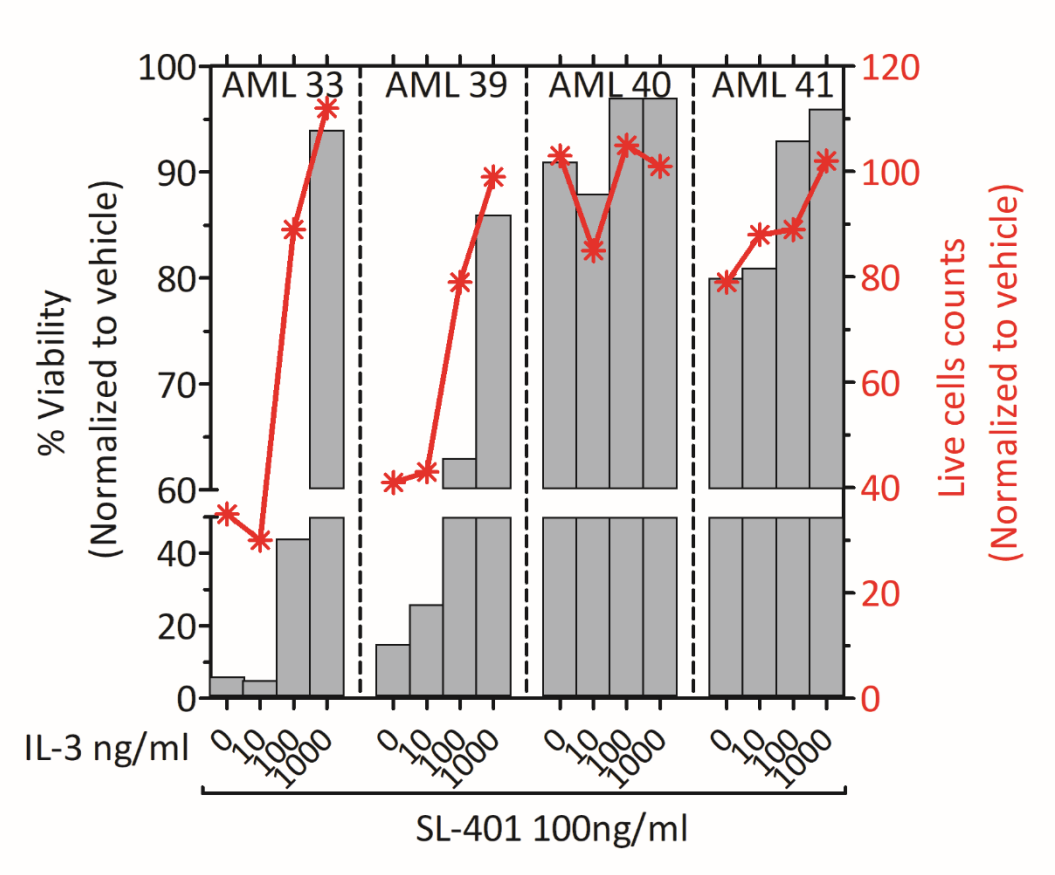
Supplementary Figure S1



CD123 expression in representative AML patient samples shown as histogram overlays using multicolor flow cytometry data. Cells were gated on live myeloid population (CD45 low/SS intermediate). Expression overlays for mature lymphocytes are also shown for representative samples.

Supplementary Figure S2





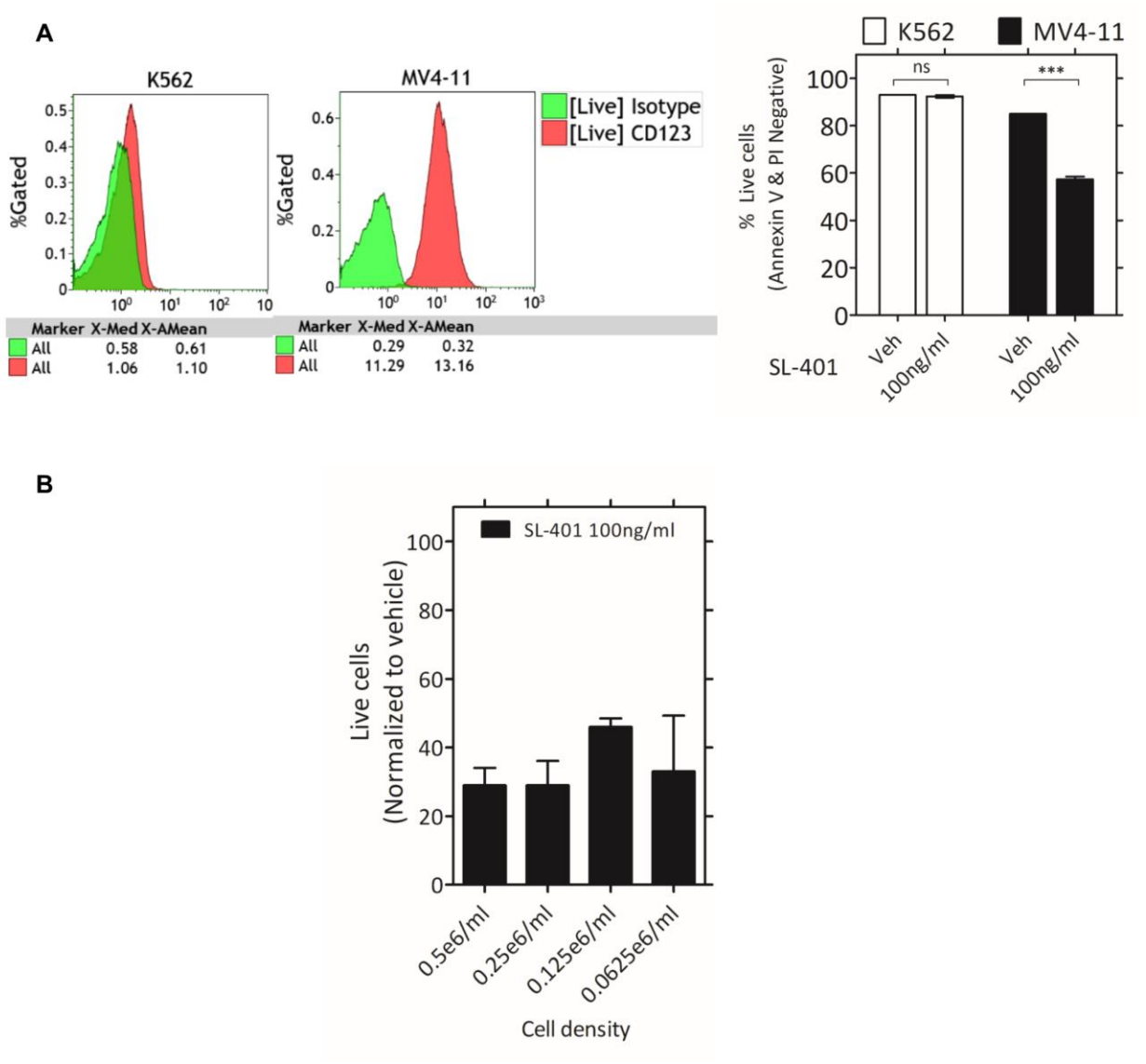
(A) Dot plots showing CD33 and CD123 expression in two representative AML patient samples determined by multicolor flow cytometry. Same AML samples as in Figure 1A.

(B) SL-401 is active against AML samples (N=6) cultured in the presence of IL-3. AML cells were cultured with vehicle or SL-401 (10ng, 100ng, 1000ng per ml) for 72 hours in RPMI1640 media with 20% FBS, antibiotics and growth factors GM-CSF, SCF and IL-3 (10ng/ml). Live cell counts normalized to vehicle control are shown.

(C) Effect of IL-3 on SL-401 mediated cytotoxicity. AML (N=7) cells were cultured in the presence or absence of recombinant IL-3(10ng/ml) and treated with vehicle or SL-401 for 72 hours and live cells were counted.

(D) Super physiological levels of IL-3 inhibit SL-401 cytotoxicity. AML cells were treated with indicated concentrations of IL-3 and after one hour dosed with vehicle or SL-401 (100ng/ml), and cultured for 72hours. Live cells in cultures were counted and normalized to their respective vehicle controls.

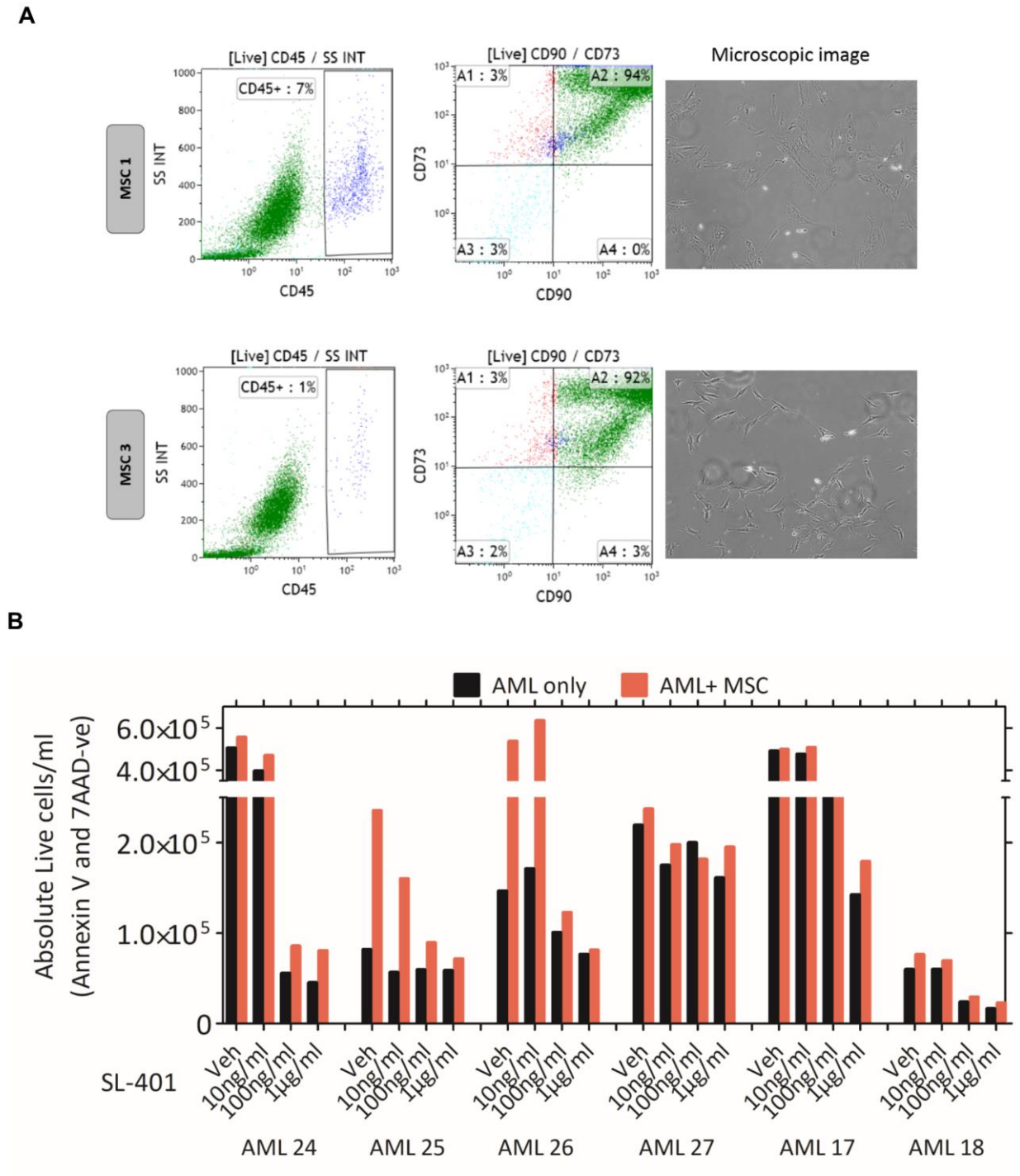
Supplementary Figure S3



(A) CD123 expression in K562 and MV4-11 cell lines and their susceptibility to SL-401 cytotoxicity. Cells ($0.5e^6$/ml) were cultured with vehicle or indicated concentrations of SL-401 for 48 hours and viability was assessed by flow cytometry.

(B) Effect of cell density on SL-401 cytotoxicity. MV4-11 cells were seeded at various cell density and treated with vehicle or SL-401 (100ng/ml) for 48 hours. Live cells in cultures were counted and normalized to their respective vehicle controls. Overall trend test across four cell densities tested revealed no significant difference after SL-401 treatment (P = 0.2861).

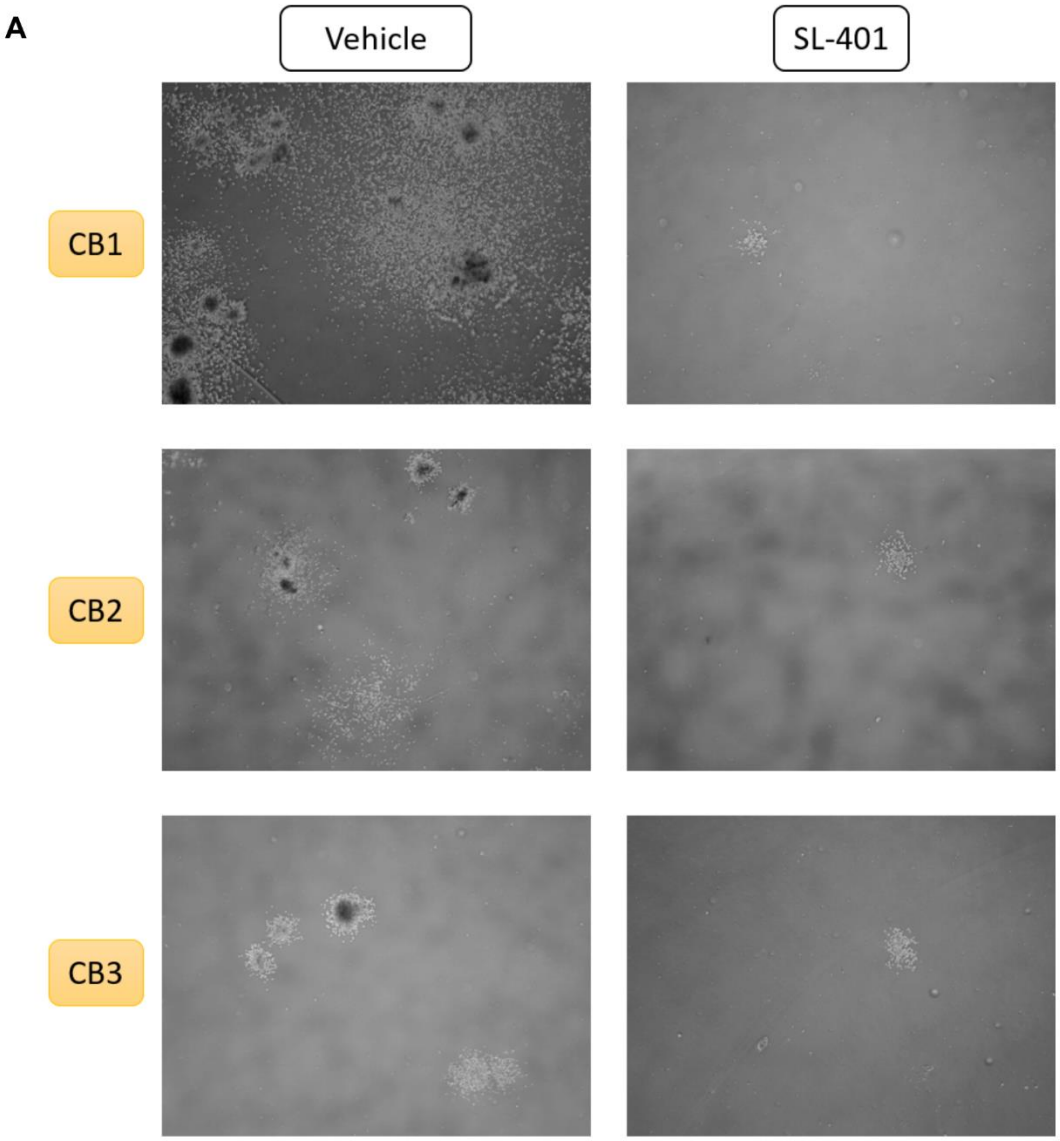
Supplementary Figure S4

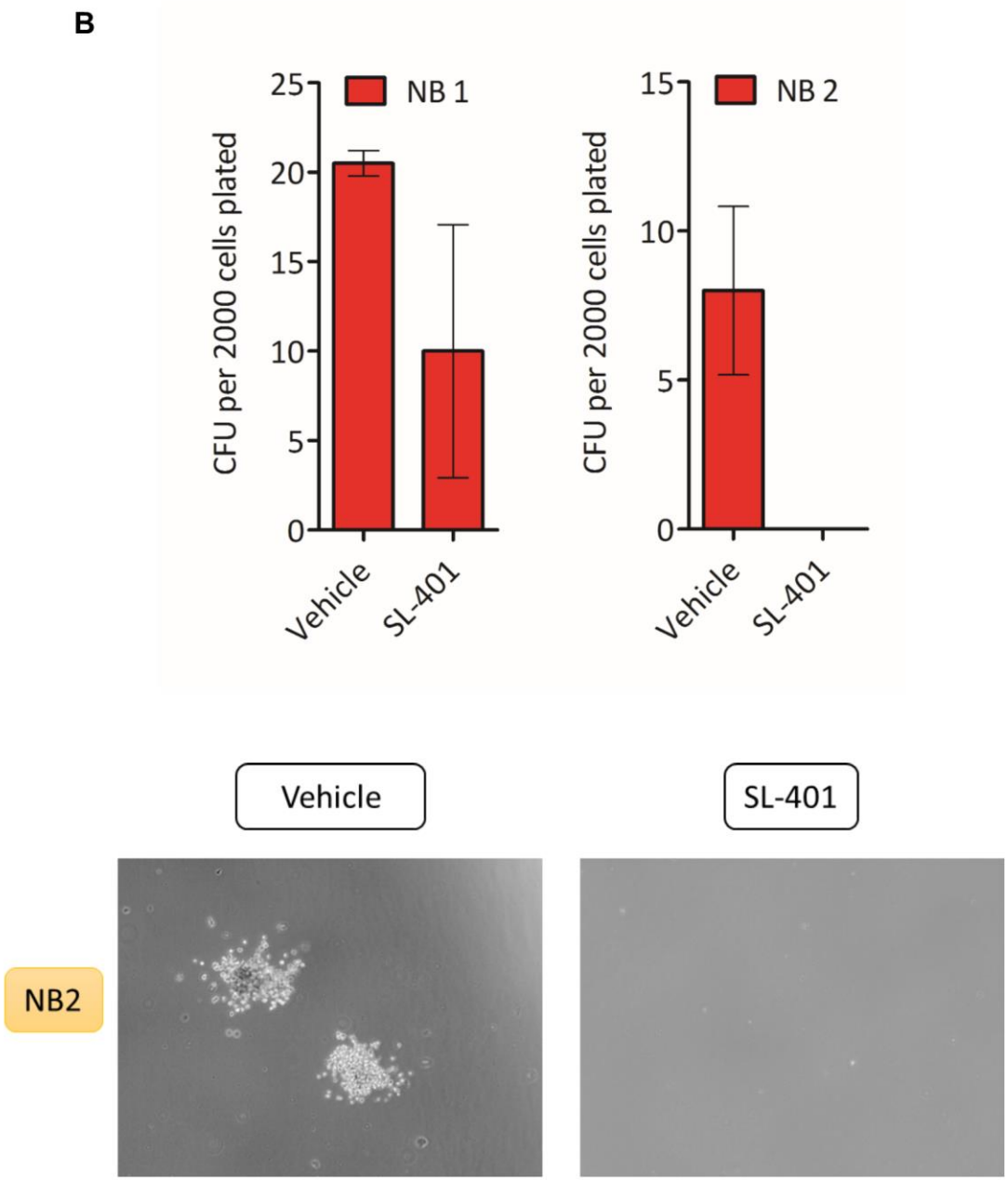


(A) Purity of AML bone marrow-derived MSC (MSC 1 and MSC3). Representative flow cytometric dot plots and phase contrast microscopic images are shown. Spindle-shaped, plastic-adherent MSC were visualized though EVOS® XL Core imaging system (PH 10X).

(B) Effect of SL-401 in AML autologous MSC co cultures (same experiment as Figure 2C). Changes in live cell concentration after 120 hours of treatment with varying concentration of SL-401 are shown.

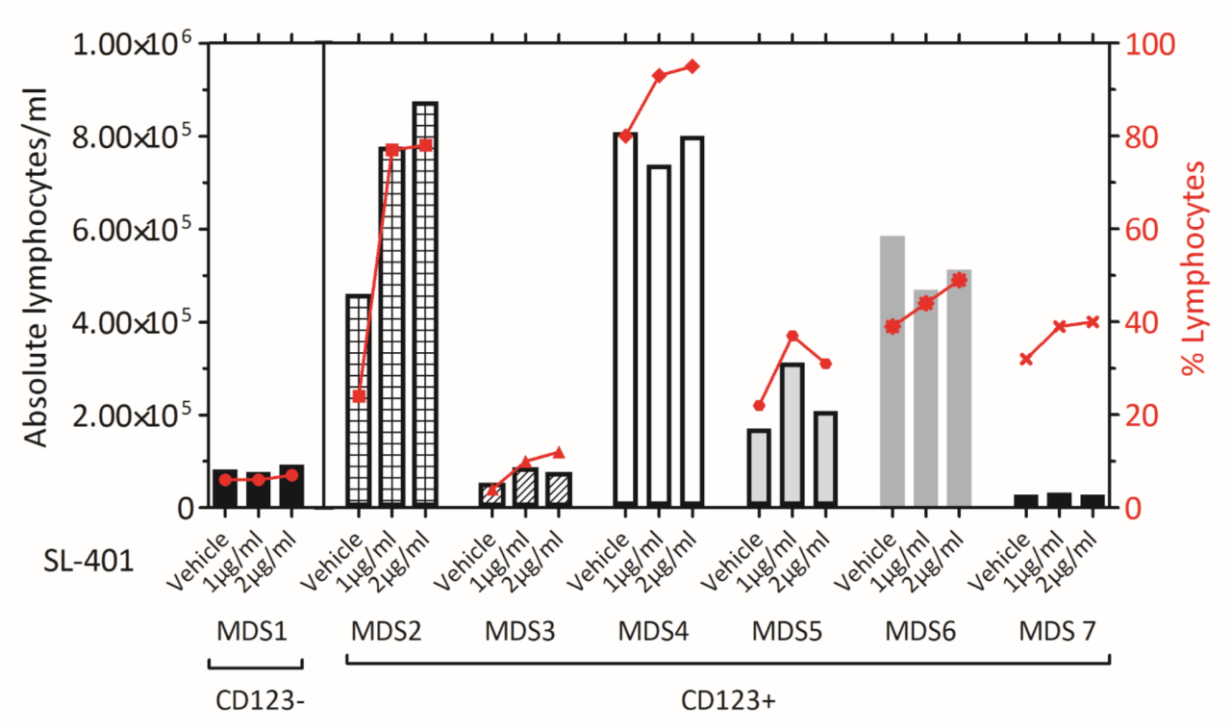
Supplementary Figure S5





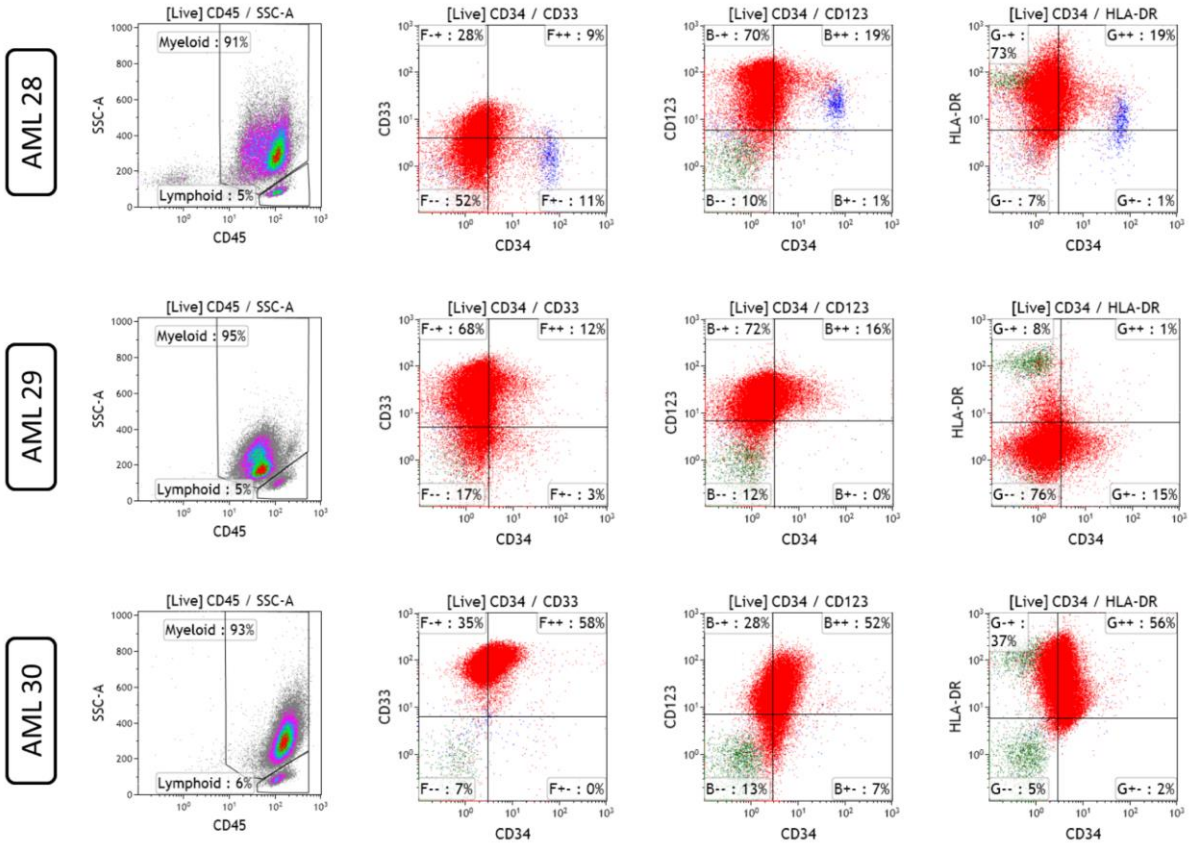
(A-B) SL-401 inhibits myeloid colony formation in $CD34^+$ cells derived from umbilical cord blood (CB) (A) and normal donor bone marrow (NB) (B). $CD34^+$ cells were positively selected from CB using CD34 MicroBead Kit Ultrapure (Miltenyi Biotec, CA, USA). NB cells were sorted using FACS for $CD34^+CD38^-lineage^-$ cells. Colonies were counted 10-14 days after plating $CD34^+$ cells with continuous presence of vehicle or SL-401(1µg/ml) in duplicates. Representative field images of plates are shown. Images were acquired using EVOS® XL Core imaging system (PH 4X) for cord blood samples and (PH 10X) for bone marrow samples.

Supplementary Figure S6



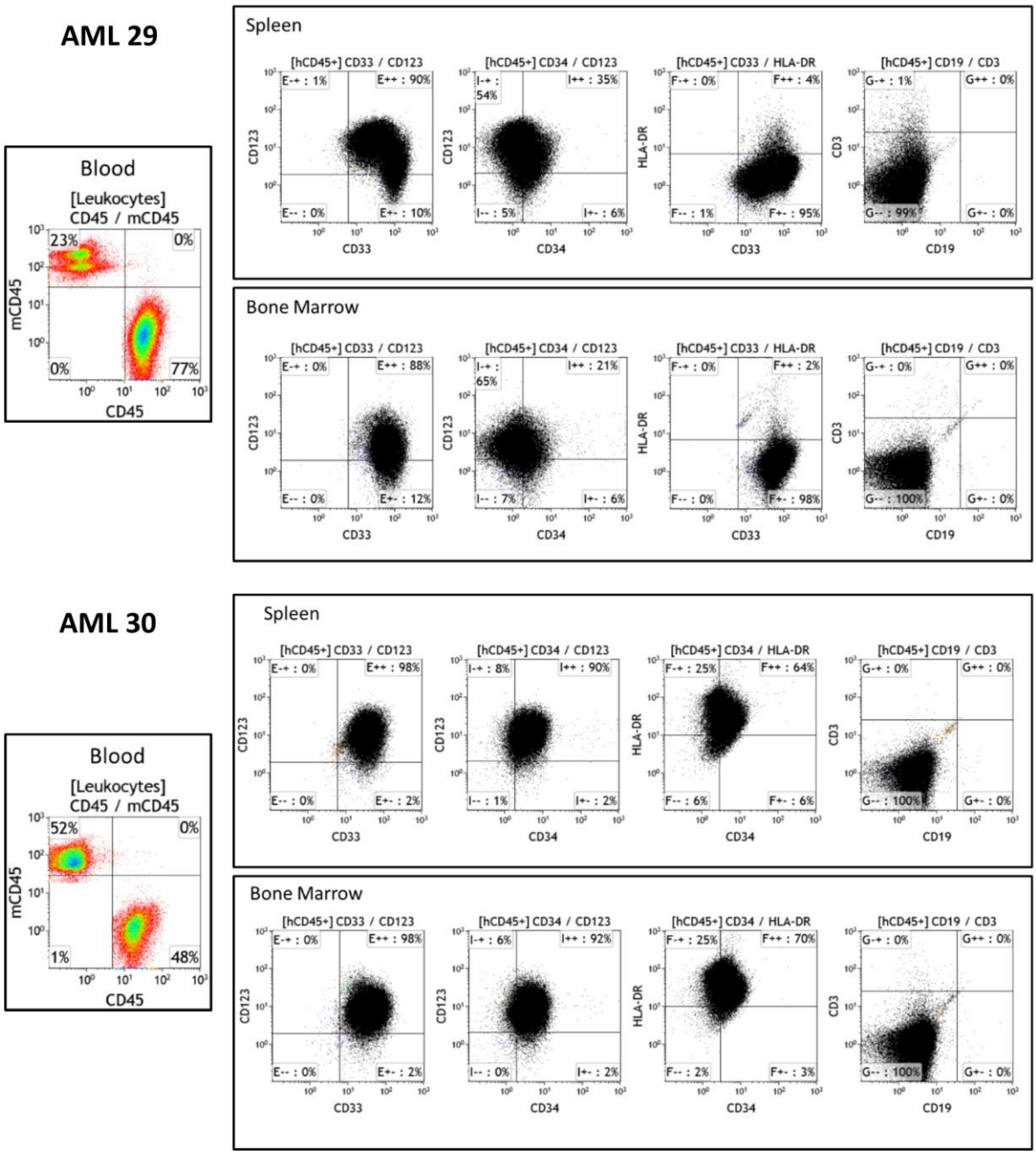
SL-401 does not reduce the lymphocyte counts in MDS. MDS samples cultured with vehicle or SL-401 (1µg/ml, 2µg/ml) for 120 hours were stained for markers CD45, CD33, CD34, CD123, and viability stain and live cells were counted. Live lymphocyte counts (bars) and % lymphocytes (red lines) are shown for each samples (N=6 $CD123^+$ MDS).

Supplementary Figure S7



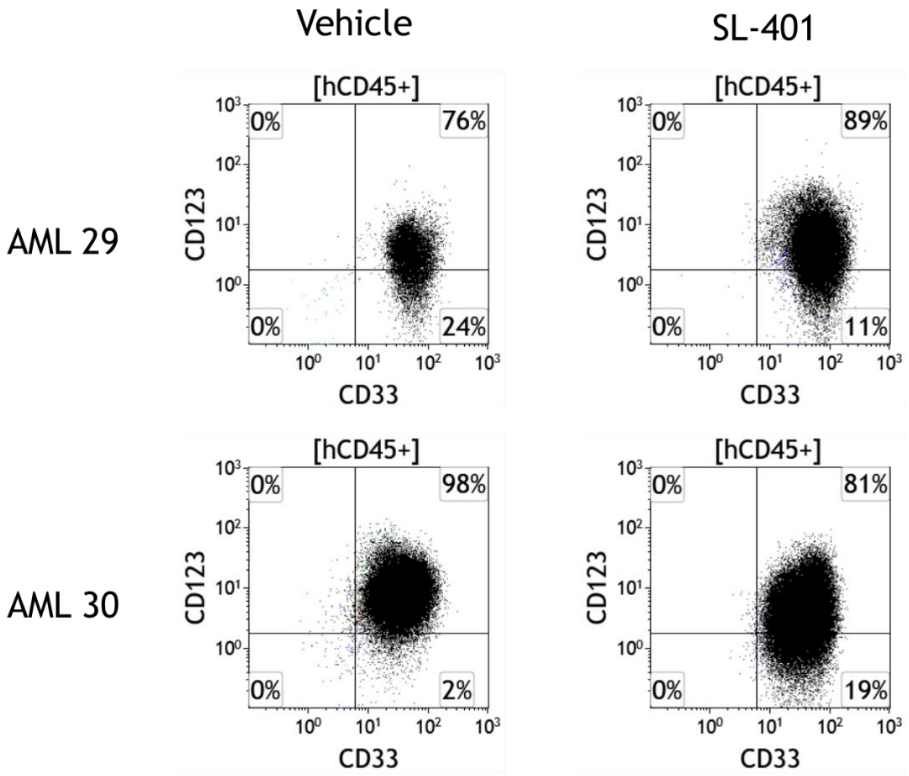
Immunophenotype of AML patient samples used to establish successful AML PDX in NRGS mice with or without pre-conditioning agent (AML 28 and AML 29 engrafted into busulfan conditioned mice; AML 30 engrafted into unconditioned mice). Color dot plots gated on live cells are shown. Most AML cells were $CD33^{+}CD123^{+}$. Low levels of lymphoid cells (green dots) were also noted.

Supplementary Figure S8



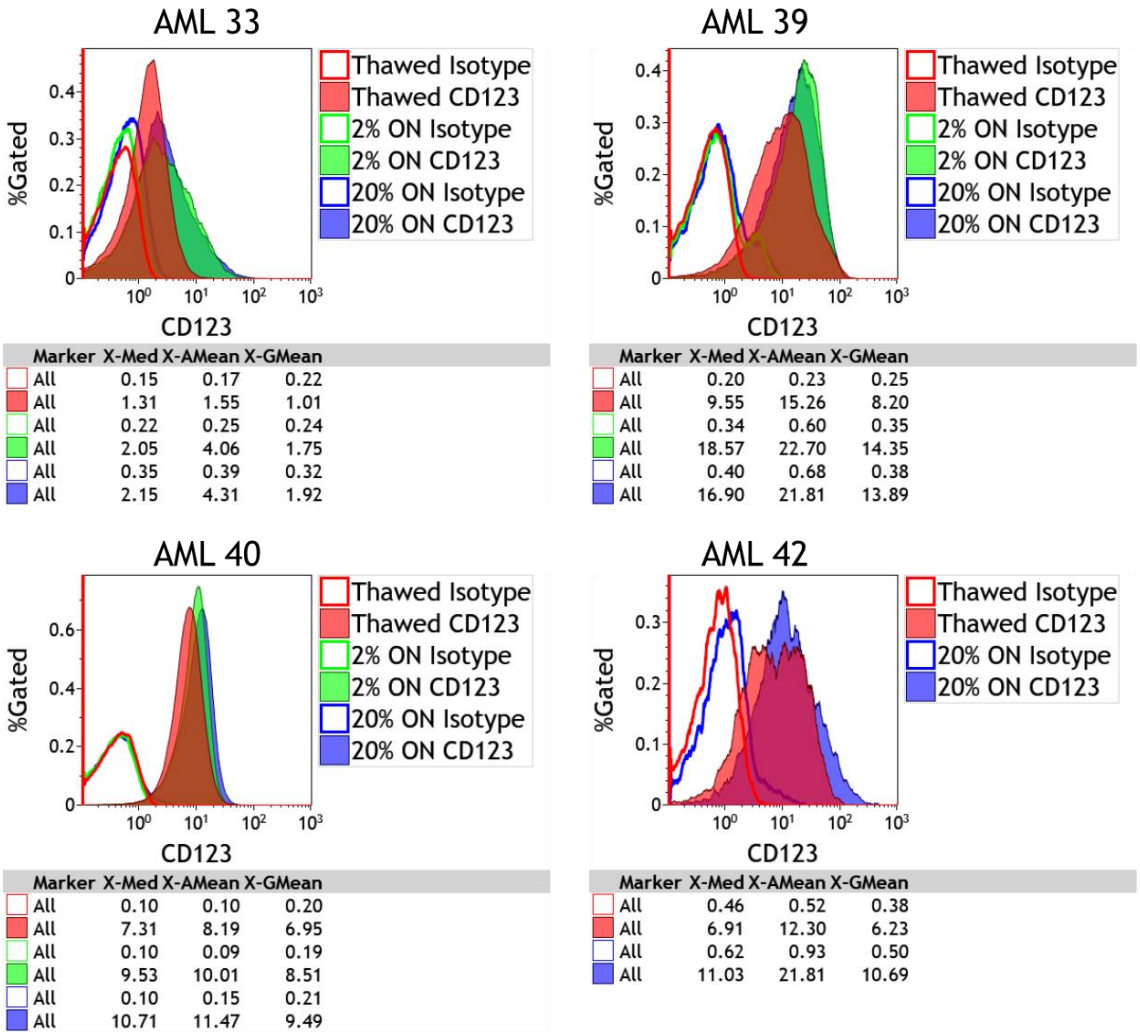
The presence of AML in the PDX mice was confirmed in spleen, bone marrow and blood using a multi-color flow cytometric panel (CD45, CD33, CD34, CD123, CD19, CD3, HLA-DR, mCD45, and viability stain). Representative multicolor flow cytometric plots showing AML in various organs of NRGS mice engrafted with AML 29 and AML 30 are shown.

Supplementary Figure S9



Representative flow cytometric dot plots showing CD123 expression in AML cells from bone marrow of AML PDX mice treated with vehicle (left panel) or SL-401(right panel).

Supplementary Figure S10



ΔMFI	AML 33	AML 39	AML 40	AML 42
Thawed (0h)	1.38	15.03	8.09	11.78
2% FBS (24h)	3.81	22.10	9.92	ND(poor viability)
20% FBS (24h)	3.92	21.13	11.32	20.88

Impact of culture procedure on CD123 expression in primary AML samples. CD123 (clone 6H6) and relevant isotype antibodies were used to test CD123 expression on AML samples that were thawed form cryovials and on same AML cultured for 24hours in RPMI base medium with 2% and 20% FBS. There was similar CD123 expression in cells cultured with 2% FBS and 20% FBS. Data is presented as overlays and ΔMFI (ΔMFI =Arithmetic Mean of CD123 - Arithmetic Mean of Isotype).

Supplementary Table 1. AML FAB subtype and cytogenetic/molecular information of primary samples used for the study.

Expt/AML	No	FAB Subtype	Cytogenetic/Mutation Analysis
Figure 1A, C			
AML 1	U-03-438	M2	NA
AML 2	U-04-370		NPM1 positive; IDH1 positive
AML 3	U-05-0471		FLT3-TKD positive; NPM1 positive
AML 4	U-06-0115		NPM1 positive
AML 5	U-06-0459		NA
AML 6	U-03-217		NA
AML 7	U-07-0466		RUNX1 positive; ASXL1 positive; SRSF2 positive
AML 8	U-07-1069		FLT3-ITD positive; NPM1 negative; IDH2 positive
AML 9	U-07-1177		NPM1 positive;TET2 positive; ASXL1 positive
AML 10	U-08-0907		NA
AML 11	U-08-1445	M3	PML-RARA (97.1%) positive
AML 12	U-09-1529	M1	FLT3 (ITD) positive; Normal chromosomes
AML 13	U-09-1670	M3	PML-RARA (100%) positive by marrow; (89%) positive by blood; FLT 3 (ITD) positive
AML 14	U-10-0762	M5a	FLT3 (TKD) positive; 11q23 (MLLba) negative.
AML 15	U-12-0045	M4	FLT3 (ITD) positive; NPM1 negative; CEBPA negative; & aberrant co-expression of CD19 & CD7
AML 16	U-13-1355	M1	FLT3 (ITD) positive; NPM1 positive; PML-RARA negative; RUNX1T1-RUNX1 negative; CBFBba negative
Figure 1B			
AML 17	U-13-1900	M4	FLT3 negative; RUNX1T1-RUNX1 negative; NPM1negative and CBFB negative
AML 18	U-14-1077	M2	Philadelphia positive; PML-RARA negative; FLT 3 negative, RUNX1T1-RUNX1 negative
AML 19	U-09-1714	M5	FLT3 negative; NPM1negative and CBFB negative
AML 20	U-12-0126		NA
AML 21	U-12-1629	M3	NPM1 positive; FLT3 negative; CEBPa negative; PML-RARA negative
Figure 2B			
AML 8	U-07-1069		FLT3-ITD positive; NPM1 negative; IDH2 positive
AML 10	U-08-0907		NA

AML 13	U-09-1670	M3	PML-RARA (100%) positive by marrow; (89%) positive by blood; FLT 3 (ITD) positive
AML 16	U-13-1355		NPM1 positive; IDH2 positive
AML 14	U-10-0762	M5a	FLT3 (TKD) positive; 11q23 (MLLba) negative.
AML 22	U-05-0032		NA
AML 23	U-09-0891	M2	FLT3 (ITD) positive; NPM positive; PML-RARA negative; Normal chromosomes
Figure 2C			
AML 24	U-09-1713	M5	FLT3 ITD negative; NPM1negative; CEBPa negative
AML 25	U-11-1361	M1	NPM1 positive; FLT3 ITD negative; CEBPa negative
AML 26	U-14-0269	M5	11q23 (KMT2Aba) positive; FLT 3 negative; RUNX1T1-RUNX negative; CBFBba negative
AML 27	U-14-1295	M5a	FLT positive (TKD) RUNX1T1-RUNX1 positive; BCR-ABL1 negative, CBFBbap negative, NPM1 negative
AML 17	U-13-1900	M4	FLT3 negative; RUNX1T1-RUNX1 negative; NPM1negative and CBFB negative
AML 18	U-14-1077	M2	Philadelphia positive; PML-RARA negative; FLT 3 negative, RUNX1T1-RUNX1 negative
Figure 5			
AML 28	U-12-1463	M5b	FLT3(ITD) positive; NPM1 positive; CEBPa negative; PML-RARA negative
AML 29	U-14-0465	M1	FLT3 (ITD) positive; RUNX1T1-RUNX1 negative; PML-RARA negative; CBFBba negative
AML 30	U-14-0270	M2	11q23 (KMT2Aba) positive; FLT 3 negative; RUNX1T1-RUNX negative; CBFBba negative
S.Figure 1			
AML 31	U-11-0888		NPM1 positive
AML 32	U-12-0162		FLT3(ITD) positive; NPM1 positive
AML 33	00252455		AML with monocytic differentiation/NA
S.Figure 2C			
AML 34	U-12-0130	M4	FLT3 ITD negative; NPM1 negative; CEBPa negative
AML 35	U-12-0954	M1	FLT3 (ITD) positive; CEBPA negative; NPM1 negative; Normal chromosomes
AML 25	U-11-1361		NPM1 positive; FLT3 ITD negative; CEBPa negative
AML 36	U-12-0793		NA

AML 37	U-13-1982	M5	FLT3 (ITD) positive; PML-RARA negativeative, BCR-ABL1 negativeative, RUNX1T1-RTUNX1 negativeative, and CBFB negative; Acute Monoblastic Leukemia
AML 38	U-13-1323		FLT3 negative; BCR/ABL1 negative;AML with MDS related changes
AML 18	U-14-1076	M2	Philadelphia positive; PML-RARA negative; FLT 3 negative, RUNX1T1-RUNX1 negative
S.Figure 2D /S. Figure 10			
AML 33	00252455		AML with monocytic differentiation/NA
AML 39	U-13-0571		FLT3(ITD) positive; NPM1 positive
AML 40	U-13-1316		FLT3 negative; BCR/ABL1 negative; AML with MDS related changes
AML 41	U-18-1060		FLT3 ITD/TKD negative; IDH1 and IDH2 negative; CEBPa positive
AML 42	U-10-0463		NPM1 positive
NA- Not available			