CLT030, a Leukemic Stem Cell-Targeting CLL1 Antibody-Drug Conjugate for Treatment of Acute Myeloid Leukemia

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Supplementary Information:
Methods &
Figures S1, Figure S2, Figure S3, Figure S4, Figure S5, Figure S6
Table S1, Table S2

Methods

RNA isolation and expression analysis

RNA was isolated with RNAeasy mini or micro kit and subjected to whole transcriptome sequencing analysis using the Illumina-HiSeq 4000 platform (Beckman Counter Genomics). Selection of the CLL1 target was based on comparing normalized mRNA transcript reads per kilo base of transcript (RPKM) in LSC of AML samples vs. other normal tissue including normal HSC, colon, heart, lung, liver, kidney, pancreases, and skin. Normal tissue RNA was obtained from a pooled of five healthy individual donors (Life Technology). Taqman analysis were done using 1 ng of RNA to generate pre-amplified cDNA and then 1 ng of cDNA was mixed with 1X Taqman gene expression master and primer and probe sets (Life Technology). The data were collected and analyzed with Taqman real time PCR (QuantStudio 6; Life Technology), and data were normalized to the average of the endogenous controls (ACTB and RPL19). Expression level was calculated for each sample relative to the average expression in normal tissues using the $2^{-_{\Delta\Delta}Ct}$ method.

Immunohistochemistry (IHC) analysis

Fresh cultured cells (adjusted to 10^5 cells/100 mL medium) were deposited onto a slide, fixed in 10% formalin at room temperature for 15-30 min, transferred into PBS and kept at 4°C for IHC. The IHC assay was applied on a Ventana Discovery Ultra platform. HEK293 and HEK293-CLL1 were stained with both control IgG_1 and rabbit polyclonal anti-CLL1 antibodies (R&D Systems) to validate the antibody for IHC analysis. This antibody was then used to measure the CLL1 expression in multiple organ tumor and normal adjacent tissue array (US Biomax, Inc.).

Humanization of lead anti-CLL1 antibody

The lead anti-CLL1 antibody was humanized using the CDR/framework replacement method. The VH and VL sequences of anti-CLL1-antibody were compared to databases of human VH and VL germline sequences from the NCBI website (http://

www.ncbi.nlm.nih.gov/igblast/) (Ye J., Ma N., Madden T.L., Ostell J.M. IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res. 2013;41:W34–W40). The databases used were IMGT human VH genes (F+ORF, 273 germline sequences) and IMGT human VL_{κ} genes (F+ORF, 74 germline sequences). The closest human germlines were noted and one was used as acceptor VH or VL sequence. The variable heavy-chain and light-chain of the mouse anti-CLL1 antibody aligned to the human antibodies using a germline human IgG database to identify a human antibody CDR/framework that best resembled that of the anti-CLL1 antibody. The CDR/framework amino residues from the mouse anti-CLL1 antibody were swapped into the closely resembling human antibody. A series of humanized anti-CLL1 antibodies were generated by changing the mouse framework residues unrelated to anti-CLL1 CDR binding, while keeping the mouse framework residues that do contribute to CDR binding to the human antibody counterpart. These candidate antibodies were screened for binding ability to CLL1 on target cells by FACS and determined the binding affinity to the purified CLL1-extracellular domain was determined using a ForteBio. Lead humanized candidates were selected with an anti-CLL1 antibody containing a minimum amount of mouse amino acid residues while maintaining binding activity (via FACS) to target cells similar to that of the original mouse anti-CLL1 antibody.

Antibody internalization

To measure internalization kinetics of the anti-CLL1 antibody upon binding to endogenously expressing CLL1 positive HL60 cells, the anti-CLL1 antibody was conjugated to Alexa488-maleimide by partial reduction of hinge-disulfide bonds as described previously (Doronina SO, Toki BE, Torgov MY, et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nat Biotechnol 2003;21:778-784). The CLL1 antibody-Alexa488 conjugate (5 mg/mL) was incubated with HL60 cells on ice for 1 h to prevent receptor endocytosis. The cells were then shifted to 37°C for 0, 0.5 h, 1h, 2 h and 5.5 h time intervals in the presence of protease inhibitors (Roche Diagnostics). These HL60 cells were then divided into two pools and one pool was used to quench cell-surface bound Alexa488-conjugate with anti-fluorescent dye antibody (Invitrogen). Both pools were then washed and analyzed by

FACS to measure both total cell binding associated Alexa488-conjugate (cell surface bound + intracellular pool) and just intracellular binding.

Determination of CLL1 copy number on AML cell lines or AML patient samples

Quantum Simply Cellular Beads from Bangs Lab (Bangs Laboratories, Inc. Fishers, IN) were used to perform receptor quantification of CD33 and CLL1 on the surface of AML blasts or AML cell lines. Staining was according to manufacturer's protocol and the standard curves were created in QuickCal® software following instruction of Bangs Laboratories.

Preparation and characterization of anti-CLL1, anti-CD33 and control IgG1 isotype antibodies and ADCs

All antibodies described in this manuscript are expressed in CHO cells and are purified using Protein-A column chromatography. All antibodies are conjugated either with CLT-D212 or PBD (SGD1910) linker-payloads and characterized as described earlier via the engineered cysteine residues to result in the homogeneous ADCs.[16] Drug-to-antibody (DAR) ratio for IgG-D212, IgG-PBD, CLT030 (CLL1-D212 ADC), CLL1-PBD ADC, and CD33-D212 ADC were in the range of 1.7-1.8.

***In vitro* colony forming unit (CFU) assay with HSCs or with AML patient samples**

Enriched $CD34^+$ cells from healthy G-CSF mPB donors or BMMC or PBMC were blocked with 5% normal human serum (Lonza, Walkersville, MD) and with serial dilutions of ADC for 1 h at 37°C. Cells from each treatment were then mixed (without removing the ADC) with MethoCult (StemCell Technologies, Vancouver BC, Canada) containing 100 U/mL penicillin, 100 µg/mL streptomycin (HyClone), 1% GlutaMAX (ThermoFisher), 50 ng/mL GM-CSF (Miltenyi, Bergisch Gladbach, Germany), 10 nM thrombopoietin mimetic (PolyPeptide Labs, Torrance, CA), 2 U/mL erythropoietin (ThermoFisher), and 10 ng/mL each of SCF, Flt3-ligand, IL-3 (CellGenix, Freiburg, Germany), IL-6, and IL-11 (ThermoFisher). MethoCult was deposited into 6-well plates in triplicate and placed in a 37°C, 5% CO_2 humidified incubator for 13-15 days.

In vitro cell cytotoxicity assays

AML cells were plated 500 to 2000 cells per well in 96-well tissue culture plate, and incubated with serially diluted amounts of ADC from 10,000 ng/mL to 1 ng/mL to or with CLL1 antibody. For the ADC killing assay, the culture media contained 5% human serum to block non-specific antibody binding to Fc receptors expressed on myeloid derived cells. Following 5 days of incubation with an ADC, cell viability was measured with CellTiter-Glo (Promega). The luminescence value for each treated sample was normalized to the values of untreated controls cells and the percentage of cell viability was plotted as a function of ADC concentration.

In vitro ADC plasma stability

CLL1-ADC was incubated in human plasma at 37 °C for 0-5 days and the total antibody levels as well as total ADC levels were monitored by ELISA (see supplemental Figure S4). The total amount of antibody (conjugated ADC and unconjugated antibody) in the plasma was measured by capturing with mouse anti-idiotypic anti-CLL1 antibody and detecting with goat anti-human-Fc-HRP secondary antibody. The total ADC was measured by capturing with mouse anti-D212 (linker-payload) antibody and then detecting with biotinylated mouse anti-idiotypic anti-CLL1 antibody and strepatividin-HRP.

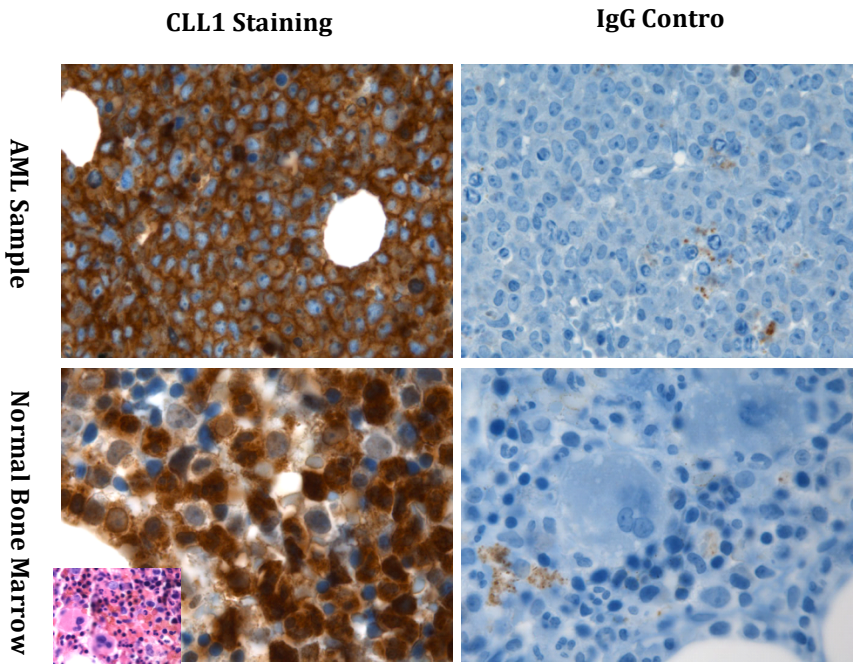


Figure S1A. IHC analysis of CLL1 expression in AML patient and normal bone marrow samples. IHC staining pattern of CLL1 with a polyclonal anti-CLL1 antibody in bone marrow samples: AML sample and normal bone marrow (H&E slide provided as inset for normal marrow). The staining in the normal bone marrow occurs in the more lineage-committed mature cells rather than the stem cells.

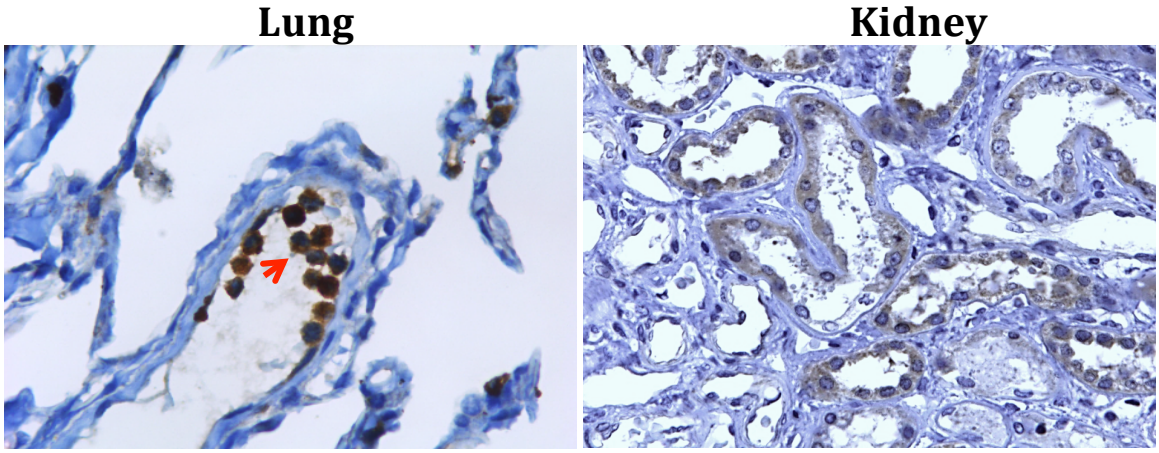
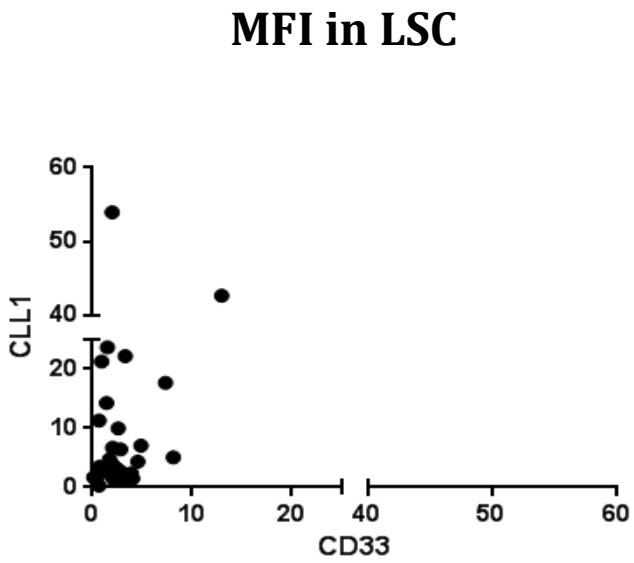


Figure S1B. IHC staining of CLL1 were observed in normal lung and kidney tissues. Lung: positive staining of CLL1 in tissue-resident macrophage was arrowed. Kidney: Expression of CLL1 in kidney luminal ductal cells is observed. The blush in the proximal renal tubule epithelial cells are favored to be non-specific staining of CLL1.

A



B

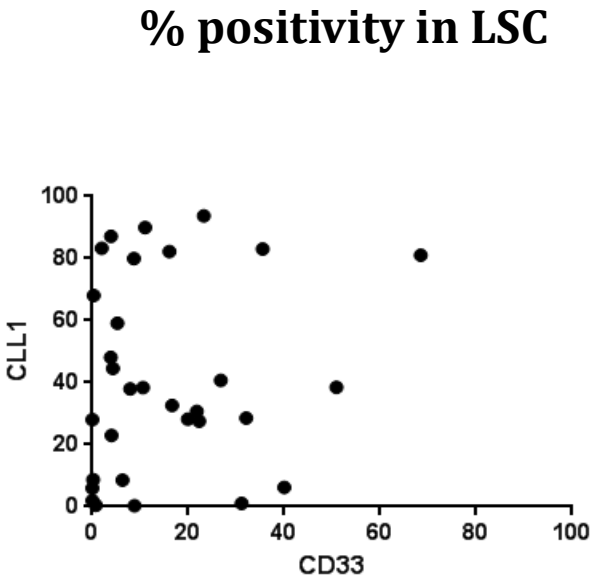


Figure S2. Relative of expression of CLL1 to that of CD33 in AML LSCs. Expression of CLL1 and CD33 was monitored by FACS analysis and their relative MFI values (**A**) or percent positive cells (**B**) in a given AML LSC were plotted.

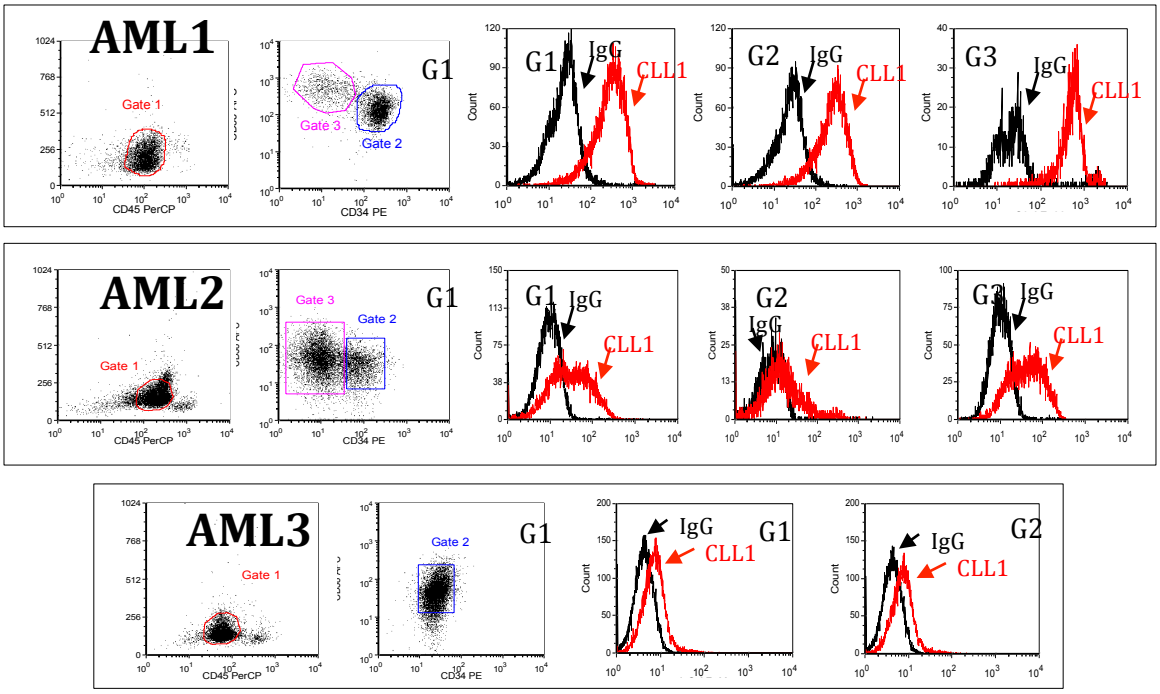


Figure S3. CLL1 expression in AML blasts. Top panel (AML1) is a strong CLL1 positive with all blast populations express the target. Middle panel (AML2) is a patient sample with heterogeneous CLL1 expression in blasts. Bottom panel (AML3) is with weak CLL1 expression.

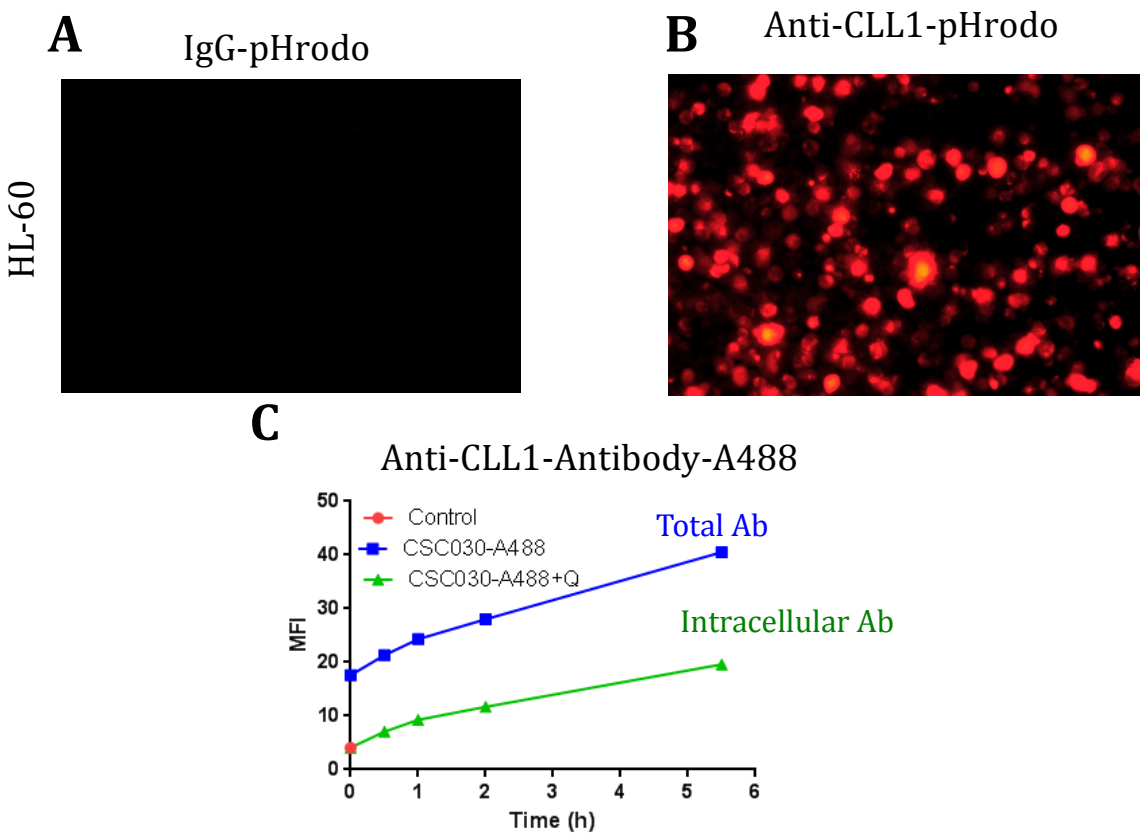


Figure S4. Internalization of anti-CLL1 antibody. Binding and internalization of Isotype IgG-pHrodo (**A**) or anti-CLL1-pHrodo (**B**) conjugates to HL60 cells at 37 °C for 4 h. **(C)** Anti-CLL1-antibody-Alexa488 conjugate (CSC030-A488) was incubated for various time intervals with HL60 cells at 37 °C and the total antibody-Alexa488 conjugate pool (cell surface + intracellular levels) was measured by FACS analysis. The intracellular pool was selectively quantitated by quenching bound cell-surface Alexa488 antibody conjugate with an anti-Alexa488 antibody (Q). Control is an isotope IgG antibody.

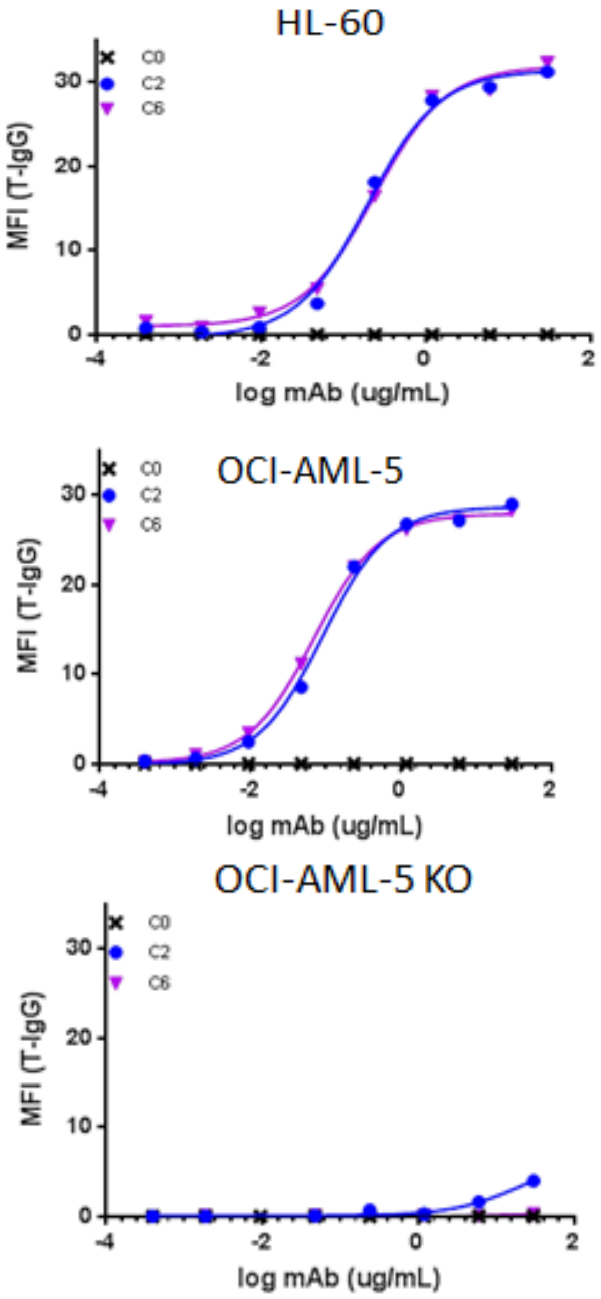


Figure S5. Anti-CLL1 Binding. Binding of chimeric anti-CLL1 antibody (blue, C2), humanized anti-CLL1 antibody (pink, C6) and isotype IgG antibody (C0) to HL60, OCI-AML5 and OCI-AML5-CLL1 knock out cells.

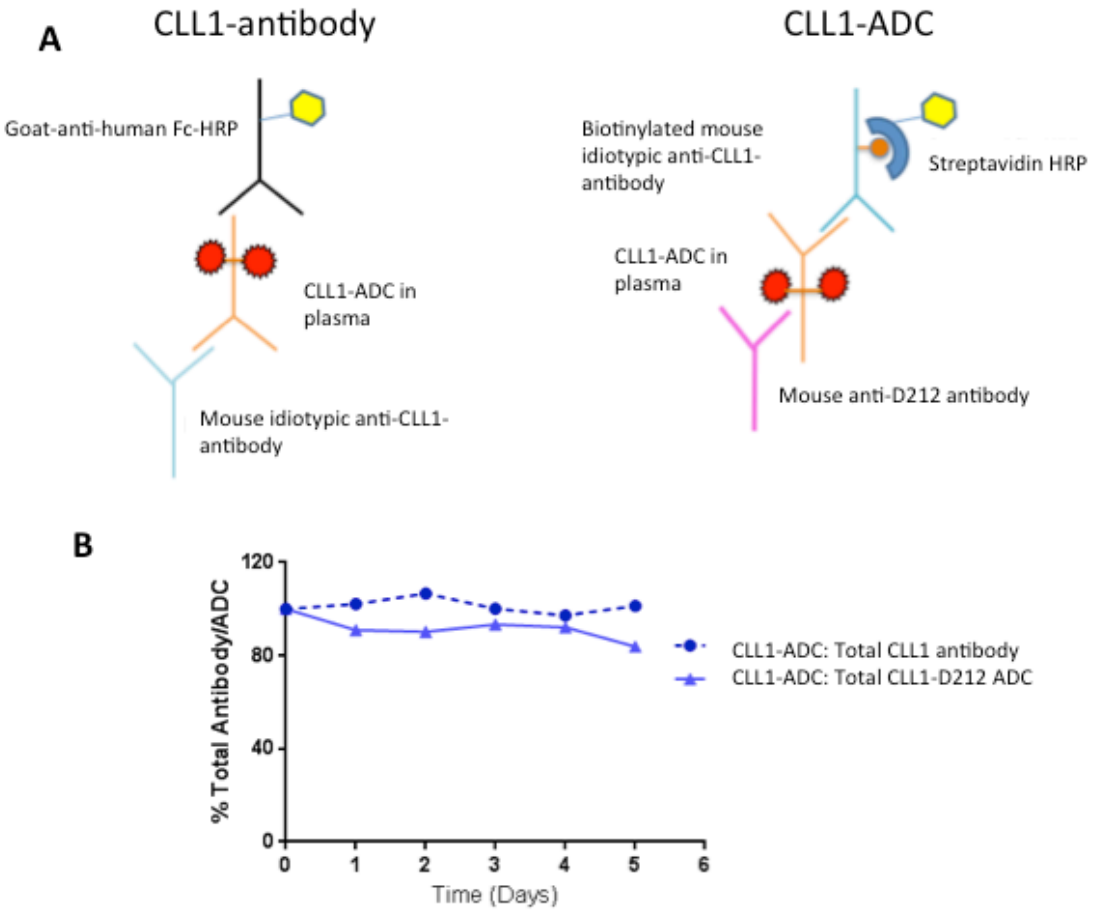


Figure S6. *In vitro* plasma stability of CLL1-ADC.
(A) Schematic of ELISA for total CLL1 antibody (**left**) and CLL1-ADC (**right**) in the human plasma. **(B)** Stability of CLL1 antibody and CLL1-ADC was tested in the human plasma and samples were analyzed as described by ELISA.

Table S1: Expression of CLL1 in human normal and cancer tissues

Tissue	Neoplastic Cell Staining	Normal tissue Staining
Bladder	None (2)	None (1)
Breast	None (2)	None (1)
Stomach	None (2)	Weak: Epithelial (1)
Colon	None (2)	None (1)
Kidney	None (2)	Moderate: luminal ductal (1)
Liver	None (2)	None (1)
Lung	Moderate (adenocarcinoma): cytoplasmic and luminal (2)	None (1)
Lymph	None (2)	Weak (Reactive Lymph): follicular dendritic cells (1)
Ovary	None (2)	None (1)
Pancreas	None (2)	None (1)
Prostate	Weak (Adenocarcinoma): epithelial and stromal (2)	None (1)
Uterus/Cervix	None (2)	None (1)

The number shown in the parenthesis denotes for number of tissue samples stained for IHC analysis.

Table S2: Analytical characterization of CLL1, CD33 and IgG1 ADCs

ADCC	DAR	Percent monomer	Endotoxin EU/mg
CLL1-D212	1.86	98%	<1.0
CD33-D212	1.70	>95%	<0.5
IgG-D212	1.80	98%	<1.0
CLL1-PBD	1.30	>95%	<1.0
IgG-PBD	1.30	>95%	<1.0

All antibodies consisted of two engineered cysteine residues per antibody, conjugated with CLT-D212 or SGD1910 (PBD) using cysteine-maleimide chemistry as described previously (Bhakta, S.; Raab, H.; Junutula, J. R.; Engineering THIOMABs for Site-Specific Conjugation of Thiol-Reactive Linkers. Methods in Molecular Biology: Antibody-Drug Conjugates, Chapter 11, Springer, 2013.), thereby resulting in homogenous site-specific ADCs.