Background

Selection of drug targets:

To identify potential therapeutic targets, we assembled a transcriptome dataset representing over 2,000 pediatric and adult leukemias including nearly 1,000 pediatric AML samples from the Target Pediatric AML (TpAML) initiative, 210 adult AML cases from the Beat AML Trial, and 707 pediatric ALL cases from St. Jude trials. We then assembled a library of targeted agents by mining multiple online databases (www.ClinicalTrials.gov and www.adcreview.com) to obtain a comprehensive list of cancer immunotherapies (primarily ADCs) that are currently undergoing development and preclinical evaluation. A total of 893 studies were manually curated and compiled into a single database; 141 unique therapeutic gene targets were identified using this approach. Therapeutic targets expressed in AML were selected by employing a minimum transcript expression threshold of 5 transcripts per million (TPM) in >50% of our AML cases. This led to the identification of 35 targets of interest for further analysis; of these 35 targets, *CD74* was among the top 10 most highly expressed genes (as ranked by median expression). The selection of targets was based solely on gene expression in leukemia and did not take into account the possible expression in normal cells (hematopoietic or other tissue).

CD74 and STRO-001 background:

CD74 is the HLA class II histocompatibility antigen gamma chain or also known as HLA-DR antigen associated invariant chain. It plays an essential role in antigen presentation by mediating the assembly and intracellular trafficking of the MHC Class II complex.[1] As one would predict, CD74 is expressed in a number of normal cells involved in antigen presentation including, B-cells, dendritic cells, Langerhans cells and macrophages. However, flow cytometry studies show that the highest level of CD74 cell surface expression is limited to leukemic blasts (Figure S1C, S6). In collaboration with HematoLogics, we are defining cell surface expression of CD74 in a large cohort of AML samples since transcriptome profiling may not correlated with cell surface expression levels. Corresponding to its role in antigen presentation, cell surface and intracellular expression of CD74 is dramatically upregulated by IFNγ stimulation. In addition, CD74 has also been implicated in several other MHC independent processes including regulation of endosomal trafficking, cell migration and cellular signaling. Cell surface CD74 serves as a receptor for macrophage migration inhibitory factor (MIF) that regulates of cell proliferation and survival.[2,3] Interestingly, CD74 expression has been associated with tumor progression and survival and

its expression has been shown to be predictive of more aggressive tumors. Upon ligand or antibody binding, CD74 is rapidly internalized making it an ideal target for ADC targeted therapy.

STRO-001 is a fully humanized, aglycosylated anti-CD74 antibody conjugated to maytansinoid, a tubulin inhibitor, by a non-cleavable linker.[4] Similar to other tubulin inhibitors maytansinoid works by inducing a mitotic arrest.[5] STRO-001 drug is in early clinical trials for treatment of multiple myeloma and B-cell lymphomas. Phase 1 trials have shown the drug to be well tolerated without ocular or neuropathic toxicity.[6] Preliminary antitumor activity has been observed in several patients with heavy treated diffuse large B-cell lymphoma.

Material and Methods

Animals

NOD/SCID/$\gamma c^{-/-}$ (NSG) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-SGM3) mice were purchased from the Jackson Laboratory. All experiments in this study utilized 6–10-week-old age and sex-matched mice that were randomly assigned to experimental groups. NSG mice transplanted with AML and ALL cell lines and NSG-SGM3 mice transplanted with cells from primary patient specimens were monitored and euthanized when they exhibited symptomatic leukemia (tachypnea, hunchback, persistent weight loss, fatigue or hind-limb paralysis). All experiments were performed after approval by Institutional Animal Care and Use Committee (protocol #51068) and in accordance with institutional and national guidelines and regulations.

Primary samples

Frozen aliquots of primary AML diagnostic bone marrow samples and primary ALL peripheral blood samples were obtained from the Children's Oncology Group and Seattle Children's, respectively. Primary cells, thawed in IMDM containing 20% FBS and 100 U/mL DNaseI (Sigma, Cat#D5025) were used for cytotoxicity assays or transplanted into NSG-SMG3 mice for *in vivo* efficacy assessment. Freshly thawed cells were used to assess CD74 expression by flow cytometry. AML cells were grown in StemSpan SFEM II (STEMCELL, 09655) supplemented with 15% BIT 9500 (STEMCELL, 09500), 2mM L-glutamine (Gibco,25-030-081), 100U/ml Penicillin/Streptomycin (Gibco, 15140122), 100ng/ml huSCF (Shenandoah, 100-04), 100ng/ml huFlt3L (Shenandoah, 100-21), 50ng/ml huTPO (Shenandoah, 100-216), 20ng/ml huIL-6 (Shenandoah, 100-10), 20ng/ml huIL-3 (Shenandoah, 100-80), 20ng/ml huGM-CSF (Shenandoah, 100-08), and 20ng/ml huG-SCF

(Shenandoah, 100-72). Human umbilical cord blood samples were obtained from normal deliveries at Swedish Medical Center (Seattle, WA). Cord blood samples were processed with red blood cell lysis buffer and enriched for CD34+ cells using CliniMACS CD34 MicroBeads (Miltenyi Biotec, Cat# 130-017-501). Cord blood CD34+ cells were then seeded onto retronectin (5 ug/mL, Takara, Cat#T100A) + Notch ligand Delta1 (2.5 ug/mL)[23] coated plates overnight in Stem Span II medium (StemCell Technologies, Cat# 09650FH) containing 50ng/mL huSCF, 50ng/mL huTPO, 50ng/mL huFLT3L, 20ng/mL huIL-3 and 10ng/mL huIL-6. All specimens used in this study were obtained after written consent from patients. The research was performed after approval by the FHCC Institutional Review Board (protocol #9950). The study was conducted in accordance with the Declaration of Helsinki.

Cell lines

K562 (ATCC, CCL-243), MV4;11 (ATCC, CRL-9591), NOMO-1 (DSMZ, ACC 542), REH (ATCC, CRL-8286) and RS4;11 (ATCC, CRL-1873) cell lines were maintained per manufacturer's instructions. K562, MV4;11 and REH were cultured in RPMI-1640 (Gibco, 11875-093) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Corning, 35-010-CV), 2mM L-glutamine, 100U/ml Penicillin/Streptomycin. NOMO-1 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine without Penicillin/Streptomycin. RS4;11 cells were cultured in Alpha MEM (Gibco, A10490-01) supplemented with 10% heat-inactivated FBS and 2mM L-glutamine, 100U/ml Penicillin/Streptomycin.

STRO-001

STRO-001 (CD74-directed ADC) and the naked antibody SP7219 were a gift from Sutro BioPharma. Drug and antibody were diluted culture medium before adding to cells or in PBS before administrating into mice.

RNA-seq analysis

To characterize *CD74* expression, we analyzed ribodepleted RNA-seq data from pediatric AML patients enrolled in COG trials CCG-2961, AAML03P1, AAML0531, and AAML1031, totaling 998 diagnostic bone marrow and peripheral blood samples. All samples selected for analysis also had CD74 flow cytometric data available. Adult AML RNA-seq data were obtained from a filtered subset of the Beat AML cohort.

Total RNA was extracted and purified using the QIAcube automated system with AllPrep DNA/RNA/miRNA Universal Kits (QIAGEN, Valencia, CA). Libraries were prepared for 75-bp strand-specific, paired-end sequencing using the ribodepletion v2.0 protocol from the British Columbia Genome Sciences Center (BCGSC, Vancouver, BC). Sequencing was performed on an Illumina HiSeq 2000/2500. Sequenced reads were quantified using kallisto v0.45.0 [7] with a GRCh38 transcriptome reference, prepared using the coding and noncoding transcript annotations in Gencode v29 and RepBase v24.01. Gene-level counts and abundances were produced using tximport v1.16.1.[8]

Adult AML RNA-seq data was obtained from a filtered subset of the Beat AML cohort. The applied criteria selected for *de novo* adult AML cases that were not treated on COG protocols; the final cohort comprised of 210 patients >18 years of age. Sample preparation and sequencing procedures for this cohort have been described previously.[9] Pediatric ALL RNA-seq data was obtained from 707 patients enrolled on clinical trial protocols at St. Jude Children's Research Hospital. The data was generated via the St. Jude Pediatric Cancer Genome Project,[10] and retrieved from the St. Jude Cloud.[11] All therapeutic data was retrieved from the U.S. National Library of Medicine clinical trials database (https://clinicaltrials.gov) and the Journal of Antibody-Drug Conjugates (https://adcreview.com) in November of 2019. Analysis of gene-level expression data was performed in the R statistical environment. Figures were generated using the ggplot2 (v3.3.5) and ComplexHeatmap (v2.9.3) packages.

Cancer immunotherapy data

The library of therapeutics used in this study was created from online databases of cancer immunotherapies (primarily ADCs) in preclinical evaluation (additional details in Supplemental information).

Flow cytometric analysis

Cell surface expression of CD74 was determined by flow cytometry using PE-labeled anti-human CD74 antibody (Invitrogen, 12-0748-42). Cells were washed in 2% FBS/PBS, blocked in 20 ug/mL human Fc block (Miltenyi Biotec, 130-059-901)/PBS at RT for 5 minutes and subsequently stained with DAPI and PE-labeled anti-human CD74 antibody and analyzed on FACSymphony equipped with FACSDiva Software (BD Biosciences). FlowJo Software was used to analyze subpopulations and CD74 expression.

***In vitro* cytotoxicity studies**

Cell lines were split 1-2 days prior to cytotoxicity assay. Primary cells were used immediately after thaw or 1 day culture and plated at a density of 20,000 cells per well on 96-well plates (Corning, 3603) with indicated dilution of STRO-001. Blocking antibody SP7219 was added at 1uM final concentration prior to addition STRO-0001. Cell viability was determined via luminescence after 3 days of continuous exposure using CellTiter-Glo (Promega, Cat# G7570). Data are normalized to untreated controls.

***In vivo* cytotoxicity studies**

Cell line-derived xenograft (CDX) models were generated by transducing NOMO-1, REH and RS4;11 cells with eGFP/ffluciferase construct (Addgene, Plasmid #104834) sorted for GFP+ expression, then transplanted into NSG mice via tail vein intravenous injection at $1x10^6$ cells per mouse. Patient-derived xenograft (PDX) models were generated by tail vein intravenous injection at $7x10^6$ cells into NSG-SGM3 mice. Mice were treated with STRO-001 at the indicated doses and time points via tail vein intravenous injection one week following leukemia cell injection. For CDX, leukemia burden was measured by bioluminescence (IVIS) imaging weekly. For PDX, leukemia burden was monitored by flow cytometric analysis of mouse peripheral blood (retro-orbital bleeds) for the indicated time points or from bone marrow aspirates at the indicated time points. At necropsy, flow cytometric analysis of leukemia infiltration in the peripheral blood and tissues was performed as described above.

Statistics

Unpaired and paired Student's t test were used to determine statistical significance and indicated in figure legends. Log-rank (Mantel-Cox) test was used to compare Kaplan-Meier survival curves between experimental groups. Statistical significance is defined as $p<0.05$.

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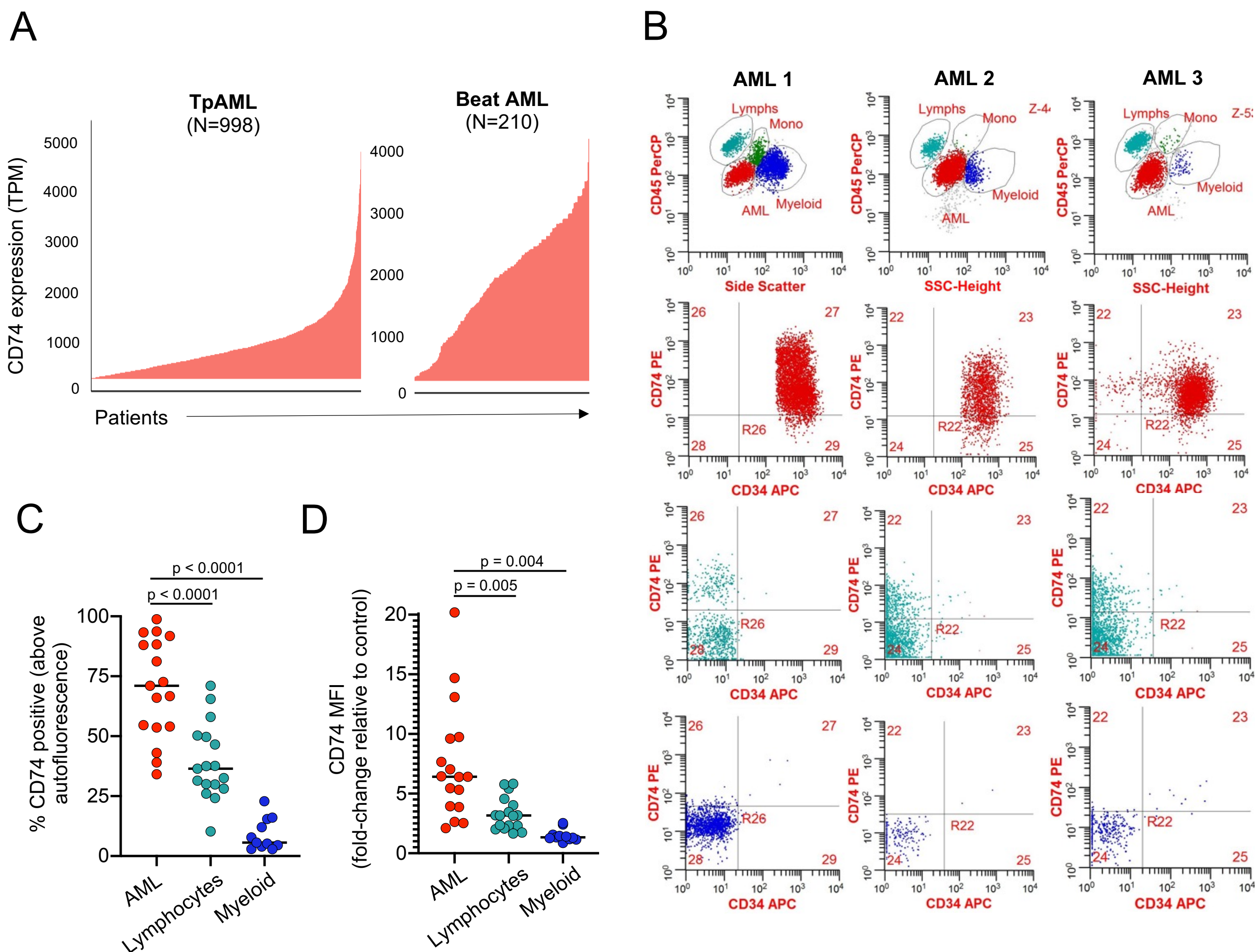


Figure S1. Transcript and cell surface expression of CD74 in AML. A. Waterfall plots showing CD74 transcript expression (TPM) from Target Pediatric AML (TpAML; 998 samples) and adult (Beat AML; 210 samples) cohorts. *CD74* was the most highly transcribed (median expression: 469.5 TPM, range: 2.2 - 4323.1 TPM) and the most prevalent; >95% of patients were found to express CD74 at ≥5 TPM (left). Evaluation of *CD74* transcript expression in the adult Beat AML cohort revealed similar high expression (median expression: 2311.2 TPM, range: 77.3 - 5223.5 TPM, right). **B.** Top, gating strategies used to identify AML cells and normal lymphocytes (Lymphs), monocytes (Mono) and myeloid cells in three CD74-positive patients based on CD45 expression and side scatter (SSC-H). Data were provided by Hematologics, Inc. **C, D.** Quantification of percent CD74+ cells (C) and the geometric mean fluorescent intensity (MFI) of CD74 expression relative to control (D) among AML blasts and their normal counterparts across N=17 patients. CD74 is expressed more frequently and at higher levels on AML blasts (median percent CD74 positive: 66.4%, range: 7.8 - 93.7%) compared to lymphocytes (37.0%, range: 7.9 - 71.0%) with rare dim expression on mature myeloid cells (median: 5.4%, range: 2.9 - 22.9%). In addition, CD74 mean fluorescent intensity (MFI relative to control) is higher in the AML subset than in lymphocytes (median 5.9, range: 1.5 - 14.7; and median: 3.1, range: 1.2 - 6.8, respectively); and lowest in myeloid cells (median: 1.3, range: 0.9 - 2.5). Autofluorescence was used as control. Paired, two-tailed Student t-test was used to determine statistical significance.

Figure S1

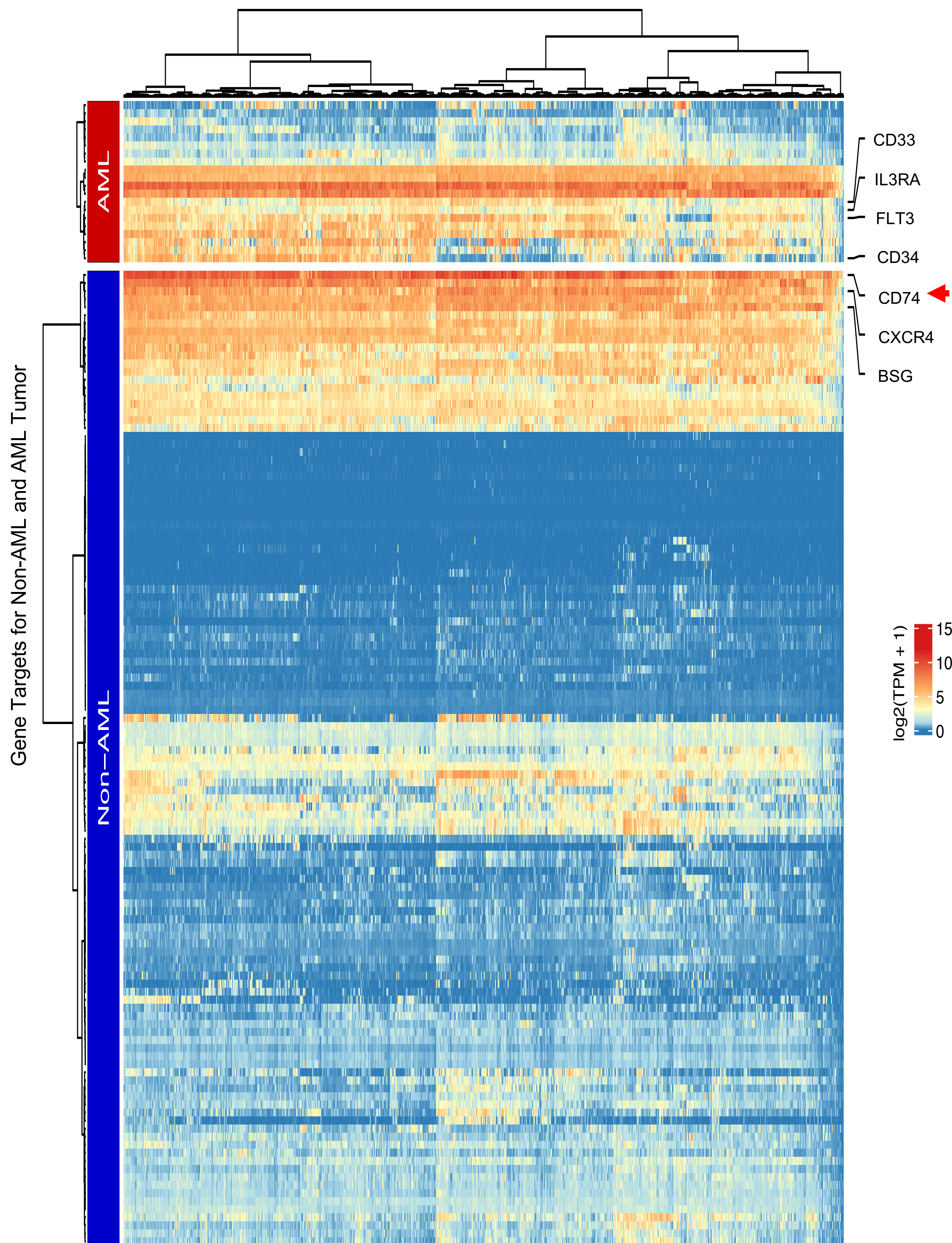
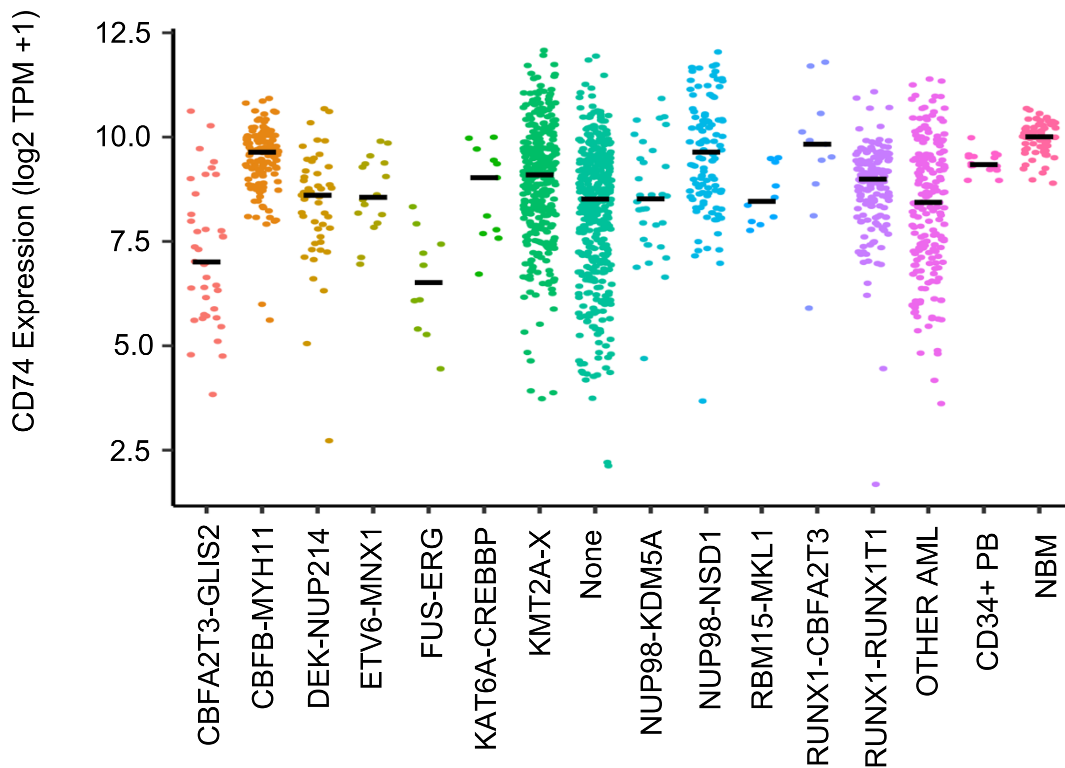


Figure S2. Expression of potential therapeutic targets in pediatric AML (TpAML). Heatmap showing expression of ADC target genes developed for AML (top) and non-AML malignancies (bottom) in TpAML dataset. Red arrow indicates CD74 target (non-AML gene target). Heatmap was generated using RNA-seq data from 993 AML samples (horizontal axis) and 141 gene targets (vertical axis). Gene targets were identified via the curated therapy database. Expression data is in log2-transformed TPM, with a value of 1 added to avoid taking the log of zero. Genes and samples were clustered via unsupervised hierarchical clustering, using Ward's method and Euclidean distances.

Figure S2



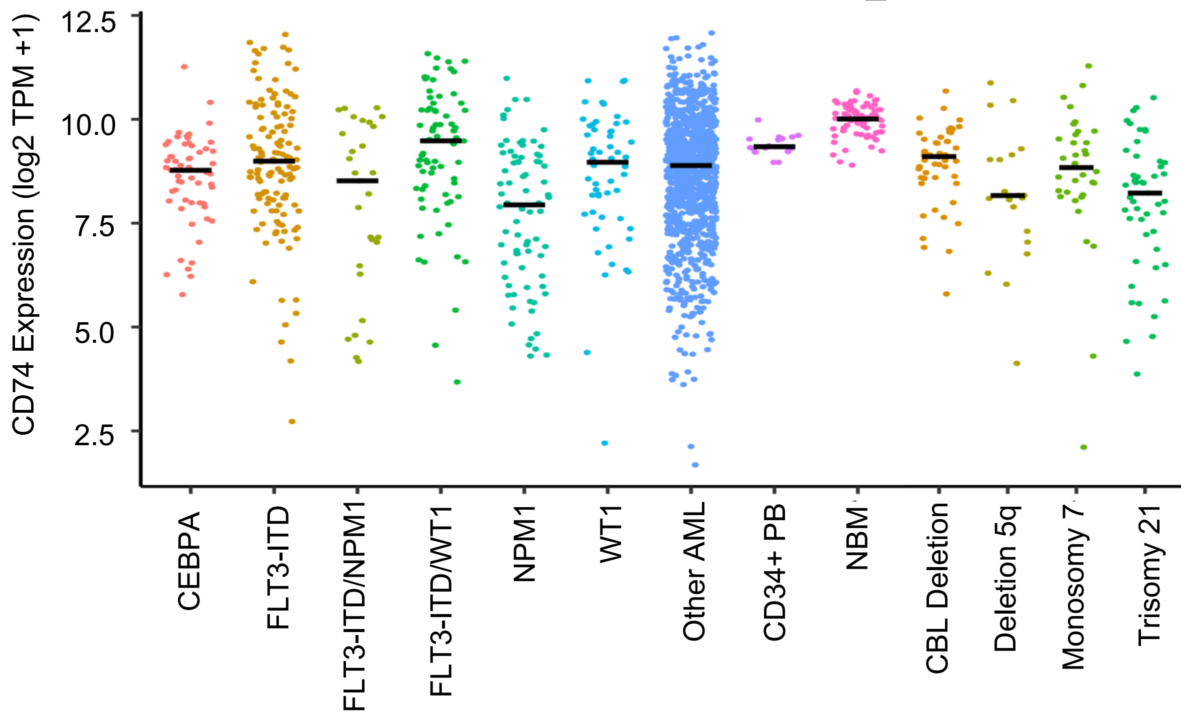


Figure S3. Transcript expression of CD74 in Pediatric AML. A. Strip plot of CD74 transcript expression in fusion positive pediatric AML from the TpAML AML cohort. CBF/GLIS AML (N=38), CBFA2T3-MYH11 (N=124), DEK-NUP214 (N=48), ETV6-MNX1 (N=16), FUS-ERG (N=10), KAT6A-CREBBP (N=11), KMT2A-X (N=327), None (N=408), NUP98-KDM5A (N=32), NUP98-NSD1 (N=104) RBM15-MKL1 (N=10), RUNX1-CBFA2T3 (N=11), RUNX1-RUNX1T1 (N=162), Other AML (N=190), normal peripheral blood (PB) CD34+ samples (N=16) and normal bone marrow samples (N=68). **B.** Strip plot of CD74 transcript expression in copy number variant (CNV) and single nucleotide variant/insertion/deletion (SNV/indels) from the TpAML AML cohort. CEBPA (N= 59), FLT3-ITD (N=127), FLT3-ITD/NPM1 (N=29), FLT3-ITD/WT1 (N=73), NPM1 (N=76), WT1 (N=54), Other AML (N=1073), CD34+ PB (N=16), normal bone marrow (N=68), CBL deletion (N=51), deletion 5q (N=21), monosomy7 (N=34), trisomy 21 (N=49). In collaboration with Hematologics, we are defining cell surface expression of CD74 in a large cohort of AML samples since transcriptome profiling may not correlated with cell surface expression levels.

Figure S3

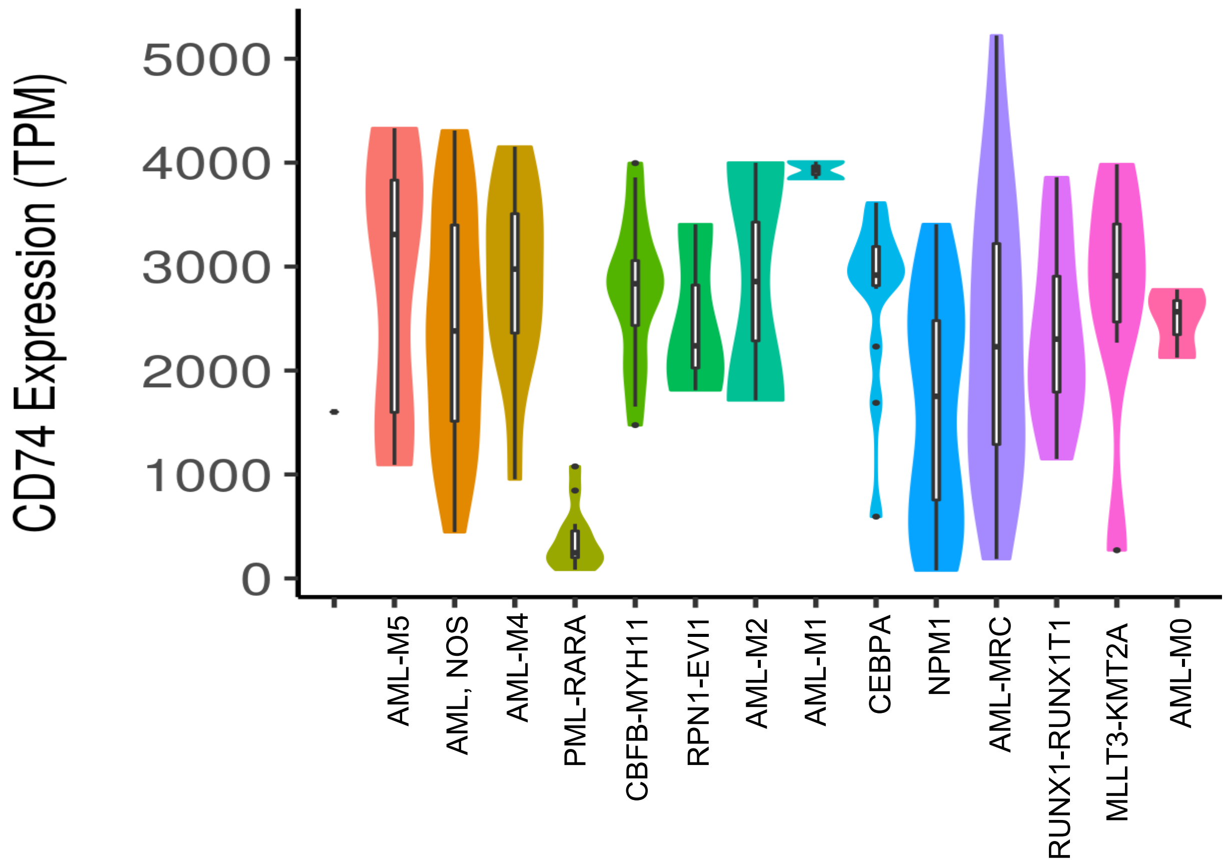


Figure S4. Transcript expression of CD74 in Adult AML. Box/violin plot of CD74 transcript expression in adult AML from the BEAT AML cohort. Acute monoblastic monocytic leukemia (AML-M5); AML, not otherwise specified (AML, NOS); Acute myelomonocytic leukemia (AML-M4), Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12), (PML-RARA); AML with Inv(16)(p13.1q22) or t(16;16)(p13.1;q22), (CBFB-MYH11); AML with Inv(3)(q21;q26.2), (RPN1-EVI1); AML with maturation AML-M2, AML with minimal differentiation AML-M1; AML with mutated CEBPA (CEBPA), AML with mutated NPM1 (NPM1); AML with myelodysplasia-related changes (AML-MRC); AML with t(8;21)(q22;q23), (RUNX1-RUNX1T1); AML with t(9;11)(p22;q23, (MLLT3-KMT2A); AML without maturation (AML-M0). Transcripts per million (TPM).

Figure S4

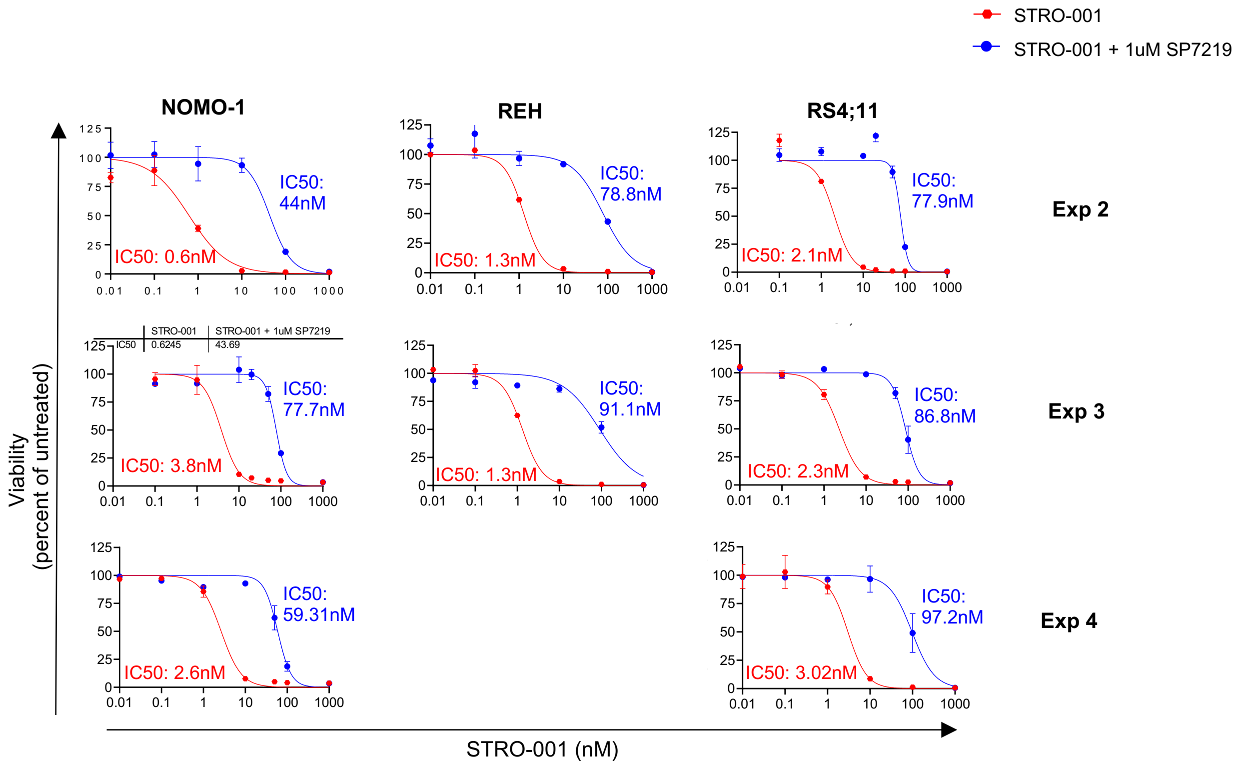
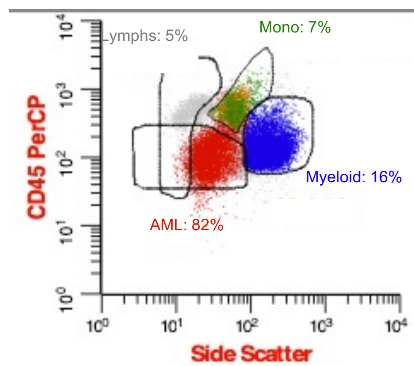


Figure S5. In vitro cytotoxicity of STRO-001 against AML and ALL cell lines. (Related to Figures 1 and 2). In vitro cytotoxicity of STRO-001 was repeated twice for NOMO-1 and REH cell lines and three times for RS4;11 cell line.

Figure S5

A



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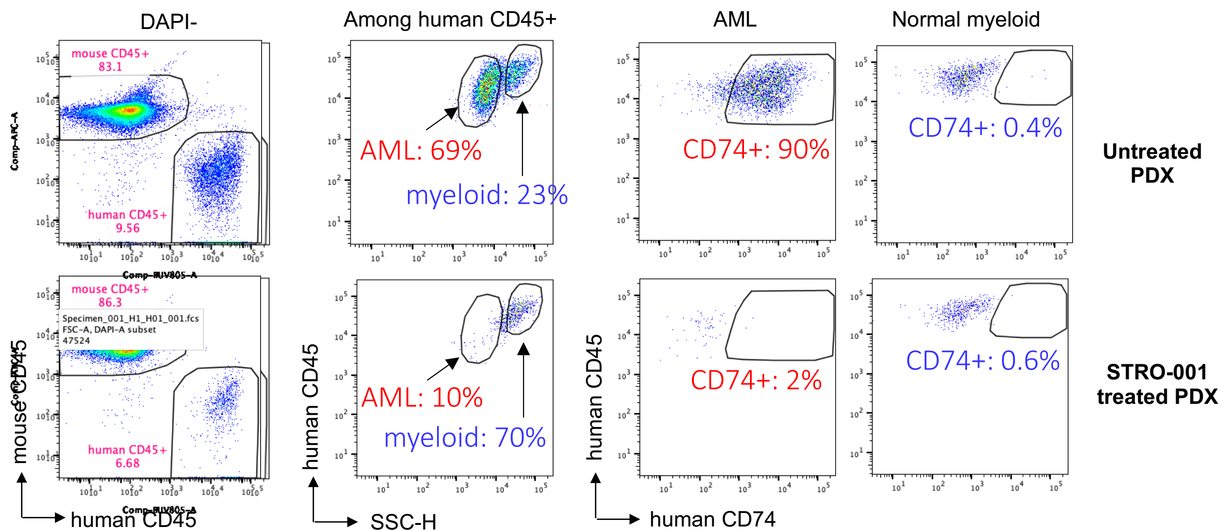

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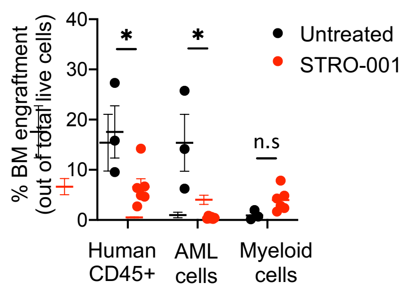

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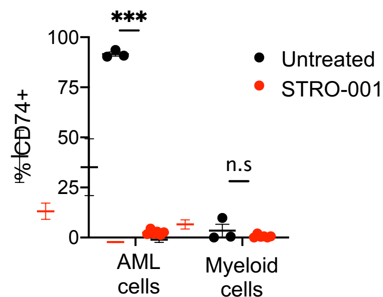

Figure S6. In vivo efficacy of STRO-001 in AML PDX model. (Related to Figure 3) **A**. Distribution of AML blasts and normal lymphocytes (Lymphs), monocyte (Mono) and myeloid subsets in patient specimen AML 7 that was used to generate the AML PDX model. Data was provided by Hematologics, Inc. **B.** Gating strategy to identify total human engraftment, AML versus normal myeloid subsets and CD74 expression among AML versus myeloid cells in the bone marrow aspirates harvested from control PDX mice (top) and PDX mice treated with STRO-001 (bottom) 4 weeks post leukemia injection. Shown is a representative mouse from each group. **C.** Quantification of total human engraftment (human CD45+ cells) and engraftment of AML and normal myeloid cells as determined by CD45 expression and SSC-H in the bone marrow of PDX mice untreated or treated with STRO-001. **D.** Percent of CD74+ cells among AML and normal myeloid cells is shown. N=3 for untreated group and N=6 for STRO-001 treated mice. Data are presented as mean +/- SEM. Statistical differences were determined by unpaired, two-tailed Student t-test.

Figure S6

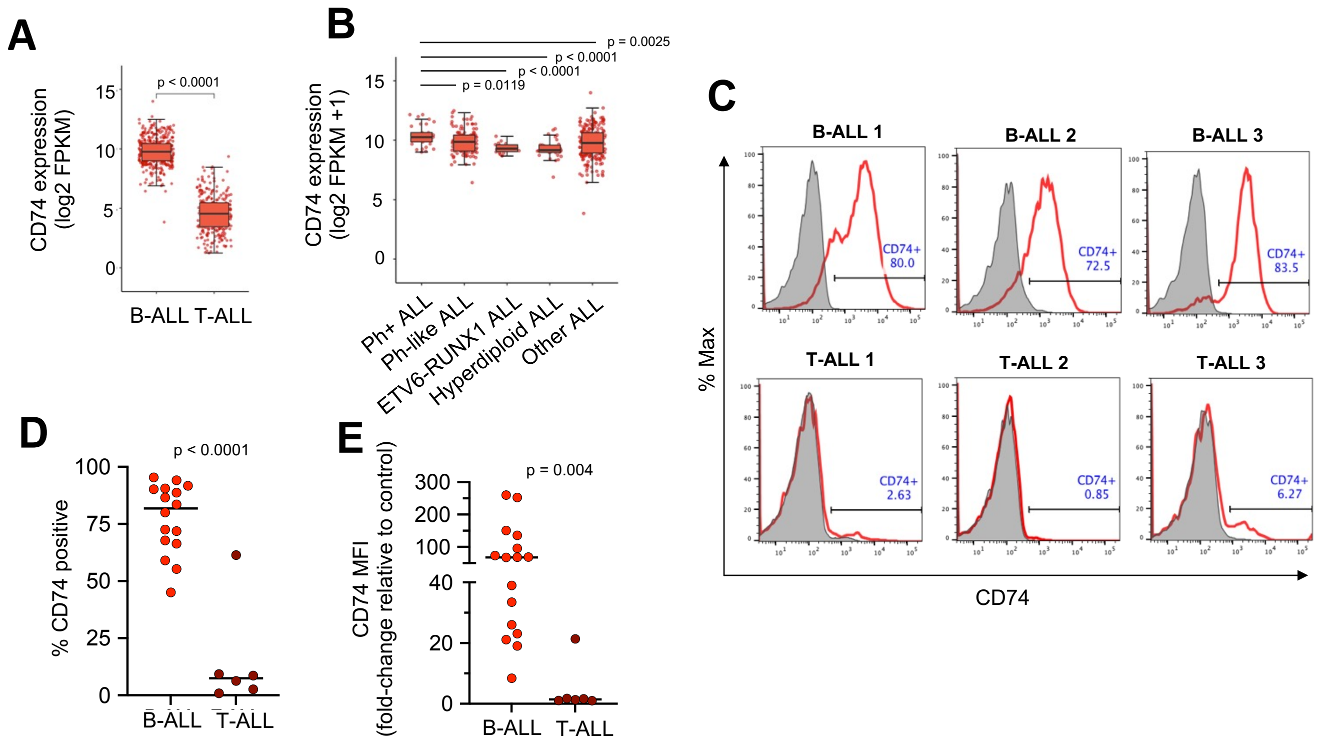

Figure S7. Transcript and cell surface expression of CD74 in ALL. A. Transcription expression of CD74 from pediatric ALL cases from St. Jude trials including B-ALL (median TPM: 861.3, range: 13.3 - 16313.6; N=418) and T-ALL samples (median TPM: 22.4, range: 1.4 – 655, Figure 1E; N=289). **B.** Transcript expression of CD74 across B-ALL with different cytogenetic abnormalities including Ph+ (N=33), Ph-like (N=106), ETV6-RUNX1 (N=20), Hyperdiploid (N=34) and Other (N=305). For both E and F, Wilcoxon tests were used to obtain p-values; in F, the Ph+ ALL group was used as a reference. **C.** Flow cytometric analysis of CD74 cell surface expression across three representative B-ALL samples (top) and three representative T-ALL samples (bottom). **D, E.** Quantification of percent CD74+ cells (H) and the geometric MFI of CD74 expression relative to isotype control (I) across B-ALL (N=16) (mean percent CD74+: 77%, range: 45 - 94%) with high MFI (mean fold change in MFI: 84, range: 8 – 260) and T-ALL (N=6) specimens. Statistical differences were determined by unpaired, two-tailed Student t-test.

Figure S7

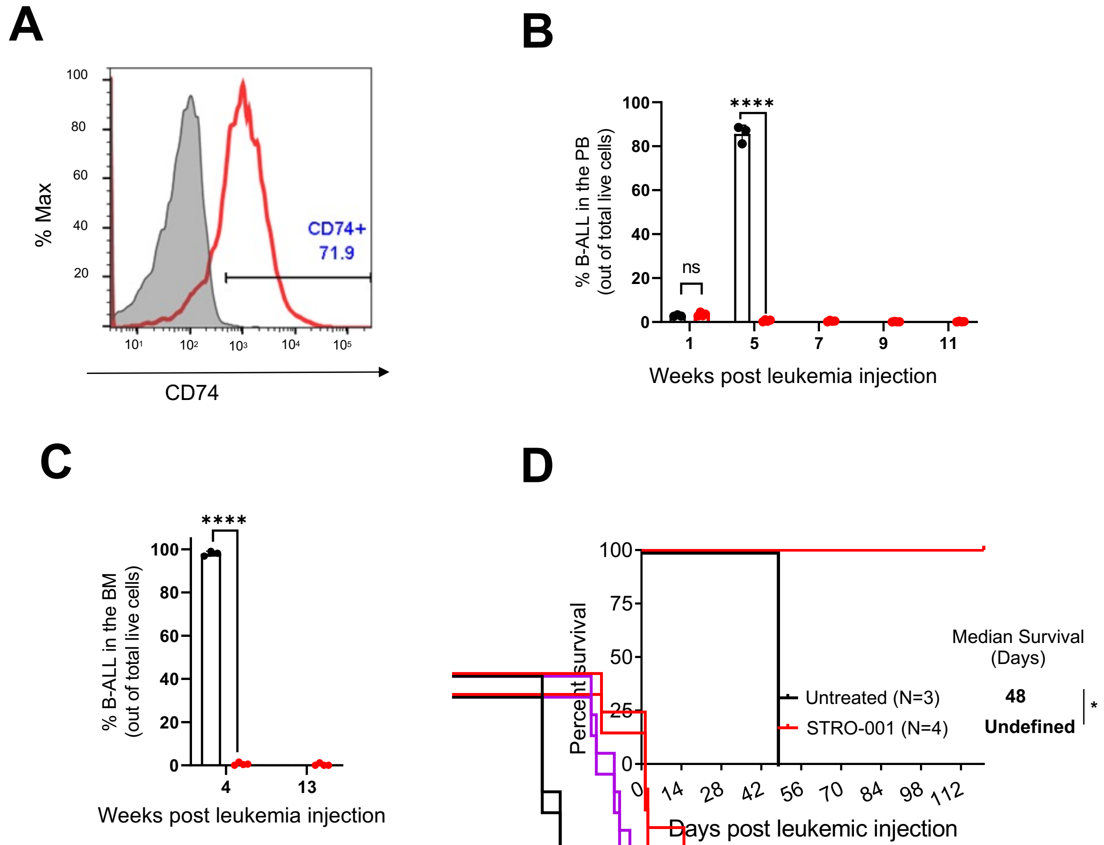

Figure S8. STRO-001 therapy demonstrates pre-clinical efficacy CD74-positive B-ALL-4 PDX model.
A. Flow cytometric analysis of CD74 expression in patient specimen B-ALL-4. **B, C.** Percent B-ALL-4 cells (huCD19+) in the peripheral blood (B) and bone marrow (C) at indicated weeks following leukemia injection **D.** Kaplan-Meier survival curves of PDX B-ALL-3 mice untreated or treated with STRO-001. Detail of B-ALL-4 PDX studies (4.5e6 cells/mouse; n=3 for untreated group, n=4 for STRO-001-treated group).

Figure S8