SUPPLEMENTAL MATERIALS FOR

Framework humanization optimizes potency of anti-CD72 nanobody CAR-T cells for B-cell malignancies

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SUPPLEMENTAL METHODS

Human Cell lines. Human cell lines were authenticated by short tandem repeat (STR) analysis. Cells were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. Cell lines used were routinely tested for Mycoplasma (Lonza, LT07-118) and were negative. SEM, NALM-6, and JeKo-1 cell lines were originally obtained from DSMZ cell bank. Modified cell lines were generated using lentivirus as described in the Method section "CAR-T Cell Production and Expansion."

Molecular Cloning and DNA Plasmids. Genes encoding the various anti-CD72 nanobody sequences were synthesized as gene fragments from Twist Biosciences (South San Francisco, CA). DNA fragments were then cloned into a lentiviral expression vector with a Gibson assembly product (NEB, E2611S). DNA sequencing was performed to confirm accuracy of the vector. This final construct was then expressed in NEB 5-alpha Competent *E. coli* (High Efficiency) (NEB, C2987H). DNA was isolated using either QIAGEN Plasmid Plus Midi Kit or QiaPrep Spin Miniprep Kit (Qiagen, 27104).

Humanization of the Framework Regions in the CD72 Nanobody. Humanized derivatives of llama NbD4 were generated based on the human variable-heavy chain framework sequence of the clinically approved anti-Her2 antibody Trastuzumab (Herceptin). The thirteen residue positions that varied between llama and human V_H framework were systematically exchanged with their human counterparts in different permutations, resulting in different NbD4 versions (NbD4-H1 through H24) that possess varying degrees of humanization. Llama residues Y37,

E44, and R45 in framework region 2 were retained as this region has been shown to be critical for the monomeric nature of nanobodies (marked in green in **Fig. 1A**). Humanized derivatives were then evaluated to resolve the optimal combination that humanizes NbD4 as much as possible, while retaining full CAR signaling efficacy.

CAR Engineering. Empty CAR, CD19, and NbD4 CAR expression plasmids utilized identical components aside from the variable extracellular binding domains (no binder for empty CAR, CD19-directed scFv with clone FMC63, or CD72 directed parental nanobody NbD4). The signaling components including the CD8 hinge and transmembrane domain, 4-1BB co-stimulatory domain, and CD3ζ signaling domain are identical to those utilized in the clinically approved CD19-directed CAR construct tisangenlecleucel. H24 nanoCAR expression plasmid included the H24 anti-CD72 nanobody binder, an IgG4 hinge region mutated to avoid Fc receptor interaction ("EQ") (24) a CD28 transmembrane domain, and a CD28 costimulatory domain. All humanized binders, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H20, H23, as well as the affinity matured nanoCAR binders NbD4.1, NbD4.3, NbD4.7, and NbD4.13 all used identical CAR backbones to the H24 nanoCAR construct. Nanobody binders were cloned into the CAR backbone plasmid using Gibson Assembly protocol. CAR expression vectors utilized a green fluorescent protein (GFP) marker for identification of successfully transduced CAR^+ cells.

Lentiviral Vector Production. Lenti-X 293T cells were transfected with each CAR expression plasmid. Lenti-X 293T cells were cultured for 2-3 days, and then lentivirus was harvested and concentrated using Lenti-X Concentrator (Takara Bio, 631232). Primary human T cells were

transduced with lentivirus, and cells underwent spinfection for 120 minutes at 244 RCF to enhance transduction efficiency.

CAR-T Cell Production and Expansion. Primary human T cells were purified from either 1) the leukoreduction filter products of anonymous healthy blood donors from Vitalant (San Francisco, CA) under an institutional review board–exempt protocol in accordance with the U.S. Common Rule (Category 4) or 2) de-identified donor leukapheresis products obtained from StemCell Technologies. $CD8^+$ and $CD4^+$ T-cell populations were isolated separately using RosetteSep Human T Cell Enrichment Cocktails (Stemcell Technologies, 15023 and 15022) and EasySep Human CD4 T Cell Iso Kit (Stemcell Technologies, 17952) and EasySep Human CD8 T Cell Iso Kit (Stemcell Technologies, 17953). CD4 and CD8 cells were thawed, cultured separately in media overnight, and then cells were counted the following day. CD4 and CD8 T cells were mixed in a 1:1 ratio. T cells were cultured in OpTmizer medium with CTS supplement (Thermo Scientific, A1048501) supplemented with 10% human AB serum (HP1022; Valley Medical), and penicillin/streptomycin (Fisher Scientific, 15-140-122) and were passaged every 2 days. For expansion, T cells were stimulated with CD3/CD28 Dynabeads (11131-D; Thermo Fisher Scientific) according to the manufacturer's instructions (20 μL of beads per 1 million T cells) for 5 days and grown in the presence of recombinant interleukin-7 (PeproTech, 200-07) and interleukin-15 (PeproTech, 200-15). Transduction with CAR lentivirus was performed 1 day after the start of bead stimulation. After the removal of CD3/CD28 activation beads, transduction efficiency was assessed by flow cytometry. CAR-T cells were labeled with intracellular GFP, so the percentage of GFP+ cells determined the percentage of CAR-T cells generated. All CAR-T constructs included an extracellular Myc tag. To isolate a pure population of CAR-expressing T

cells, anti-Myc antibodies (Miltenyi Biotec, 130-124-877) were used for enrichment via MACS column (Miltenyi Biotec, 130-042-401). CAR-T cells were then eluted, cultured in OpTmizer medium with CTS supplement, and expanded for another 4 days. CAR-T cells were cultured in the presence of IL-7 (10 ng/mL) and IL-15 (10 ng/mL), and fresh cytokines were added every 2-3 days. Flow cytometry confirmed similar transduction across all CAR-T cell constructs.

Murine Experiments. All murine experiments were performed under authorization by the UCSF Institutional Animal Care and Use Committee (Approval Number: AN194778-01) and followed all applicable ethical and veterinary guidelines and regulations. NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, Jackson Laboratories) strain mice, bred in-house at the UCSF Laboratory Animal Research Center, were used for all experiments. A mixture of male and female NSG mice, 6 to 10 weeks old, were infused via tail-vein injection with one million B-ALL tumor cells including the SEM cell line, previously modified to stably express luciferase. For JeKo-1 studies, 5e5 effLuc-labeled tumor cells were similarly injected into mice. Tumor burden was quantified via non-invasive bioluminescence imaging (Perkin Elmer In Vivo Imaging System, Caliper Life Sciences) using whole body region of interest gating. Mice were distributed to different experimental arms such that each arm had equal initial tumor burden. One day after distribution, mice received either 1.5e6 or 3e6 CAR-T cells via tail-vein injection. All mouse experiments were conducted with 1:1 CD4:CD8 CAR-T cells. Symptomatic disease was deemed the survival endpoint.

Affinity Maturation of anti-CD72 nanobodies based on NbD4 sequence. To improve the affinity of nanobody clone NbD4, we expressed a site-saturation library of NbD4 variants, fused to the Aga2 protein on the surface of yeast and performed selections for variants with increased affinity for CD72. We generated overlapping oligos that together recapitulated the entire NbD4 sequence, but that incorporate dNTP analogues in every residue position of all three CDR regions while leaving nanobody framework regions unmodified. Assembly of the library using overlap-extension PCR generated a variant library that at most generates one mutation per CDR at a time, resulting in a "soft" mutational variant library. Transformation into the EBY100 yeast strain using electroporation resulted in ~5e8 yeast transformants that were expanded for subsequent yeast display selections. We performed four equilibrium sorts with increasing stringency, starting at a 10nM concentrations of recombinant CD72 protein and ending at 100pM, using a combination of MACS and FACS sorting to select higher affinity clones. In order to isolate the highest affinity clones with a low off-rate, we performed two additional selections using 6-hr and 10-hr off-rate sorts with stringent FACS gating on the top 1% of binders. Yeast clones were isolated and sequenced, revealing convergence of key amino acid substitutions including relaxation of I33 residue identity in CDR1 and increased aromaticity in CDR2 represented by either A50W, I51W, or A52F substitutions that together accounted for a significant increase in binding affinity.

Expression of CD72 Fc-Fusion and Nanobody Fc-Fusion. DNA encoding the CD72 extracellular domain (amino acids 117–359) was polymerase chain reaction–amplified from a plasmid obtained from the Human ORFeome collection (hORFeome 8.1) and cloned into a mammalian expression vector, fused to the C-terminus of a human constant CH2–CH3 domain

(Fc domain), along with a N-terminal Avidity AviTag to facilitate site-specific biotinylation during expression. For expression, 30 μg of plasmid was transiently transfected into Expi293F cells (A14527, modified to stably express ER-localized BirA; Thermo Fisher Scientific) using polyethyleneimine (Transporter 5, 26008-5; Polysciences) at a 4:1 polyethyleneimine:DNA mass ratio. Cells were cultured in Expi293 Expression Medium (A1435101; Thermo Fisher) supplemented with 100 μmol/L biotin for 5 to 7 days to allow for protein expression and biotinylation. To purify recombinant protein, cells were pelleted and the supernatant that contained the protein was recovered, filtered, and pH adjusted with PBS (pH 7.4), prior to loading onto a HiTrap Protein A HP antibody purification column (29048576; Cytiva) to capture the CD72 Fc-fusion protein. The column was washed with PBS, and the protein was eluted with 0.1-mol/L acetic acid, then buffer exchanged into PBS using an Amicon Ultra-4 10K device (10,000 MWCO; UFC503008; EMD Millipore). Non-biotinylated nanobody-Fc fusions were similarly purified after expression in unmodified Expi293 cells lacking BirA expression. The concentration of Fc-fusion protein was determined by A_{280} by NanoDrop (Thermo), and molecular weight was confirmed by SDS-PAGE.

CAR-T *In Vitro* Cytotoxicity Assays. Cytotoxicity assays were conducted by mixing target cells with CAR-T cells for 24 hours using the indicated E:T ratios as described in each experiment. For measuring cytotoxicity by bioluminescence with target cell lines stably expressing effLuc, 150 μg/mL of d-luciferin (Gold Biotechnology, LUCK-1G) was added to each sample, incubated for 10 minutes at room temperature, and then read using a GloMax Explorer Plate Reader (Promega). Percent viable cells were normalized to the bioluminescence of tumor cells incubated with untransduced T cells at the corresponding E:T ratios or tumor cells

only. Experiments were performed with $n = 3$ to 6 technical replicates. For tumor re-exposure experiments, CAR-T cells were plated with tumor cells stably expressing effLuc at the indicated E:T ratios in each figure. After 24 hours, 1e5 target cells were added to the remaining CAR-T cells every 24 hours for the indicated number of times in each experiment. Fresh media was added, without IL-7 and IL-15 supplementation, to every tumor rechallenge.

Flow Cytometry. Staining of the cells was performed with either 1e5 or 5e5 total cells per sample unless otherwise noted. The manufacturers' recommended amount of antibody was titrated, and an appropriate amount of antibody was used in 100 uL total volume of fluorescence-activated cell sorting (FACS) buffer (PBS + 5% FBS) for 30 min prior to washing with excess FACS buffer. Samples were immediately analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter). To determine the CAR-T cell memory/stem cell-like characteristics, FlowJo software was used to gate on GFP+ CAR-T cells. The following definitions were used for the various CAR-T immunophenotypes: naïve T cells (CD45RA+/CD62L+), central memory T cells (Tcm: CD45RA-/CD62L+), T-effector memory RA (TEMRA) cells (CD45RA+/CD62L-), and effector memory T cells (Tem: CD45RA-/CD62L-). Antibodies used for flow cytometry are listed in **Supplemental Table 1**.

Quantitative Flow Cytometry. The cell surface quantification of immunotherapy targets CD72 and CD19 on tumor cells was used with Quantum™ APC MESF beads (Bangs Laboratories, 823). When mice with JeKo-1 tumor were sacrificed, spleens were isolated, crushed, and gently filtered through a 100-micron filter. Red blood cell (RBC) lysis buffer (Fisher Scientific,

NC9067514) was used to remove residual RBCs. Residual tumor cells were then washed with FACS buffer (D-PBS + 5% FBS), and antibodies against CD19 and CD72 were added for flow cytometry analysis. Quantum™ APC MESF beads were also included to determine the number of CD19 or CD72 molecules per cell. These beads contain standard numbers of fluorophore molecules per bead to generate a standard curve, and then using the median fluorescence intensity (MFI) and inserting this number into an equation from Bangs Laboratories, the number of CD19 or CD72 molecules per cell was determined.

Incucyte Live-Cell Killing Assays. Healthy donor-derived CAR-T cells were generated against CD72, CD19, or had an empty CAR construct with no antigen binding domain. CAR-T cells were co-cultured against SEM or JeKo-1 cells at the following E:T ratios: 1:1, 1:3, and 1:10. 1e5 tumor cells were used in each well, and the number of CAR-T cells varied depending on the E:T ratio in a flat-bottom clear 96-well plate. 200uL of OpTmizer media (same as above) was used per well. Loss of mCherry-positive tumor cells was used to determine cytotoxicity. Data were analyzed using the *Incucyte* Live-Cell Analysis system (Sartorius). Data were normalized using a cytotoxicity index, in which all data were normalized to the initial time point and plotted over time. Co-culture plates were incubated for 4-5 days in the Incucyte, and images were collected every four hours.

CAR-T Cytokine Release Assays. Various CAR-T constructs were cocultured with target cells at a 1:1 E:T ratio, and after 24 hours cells were centrifuged, supernatant was isolated and then snap frozen in liquid nitrogen. Cytokine samples were diluted 1:1 in RPMI + 20% FBS + 1%

pen/strep. Eve Technologies used Luminex xMAP technology for multiplexed quantification of 14 human cytokines, chemokines, and growth factors. The multiplexing analysis was performed using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Fourteen markers were simultaneously measured in the samples using Eve Technologies' Human High Sensitivity 14-Plex Discovery Assay® (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 14-plex consisted of GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-23, TNF-α. Assay sensitivities of these markers range from 0.11 – 3.25 pg/mL for the 14-plex. Individual analyte sensitivity values are available in the MilliporeSigma MILLIPLEX® MAP protocol.

Bulk RNA-sequencing. CD8/CD4 CAR-T cells were generated as described above. Bulk RNA was isolated from CAR-T cells before tumor exposure, and after tumor exposure. For CAR-T cells (empty CAR, CD19, and H24) that were exposed to SEM tumor cells, a cytotoxicity assay was set up with a 1:1 E:T ratio. After 24 hours of exposure to SEM, CAR-T cells were isolated with MACS enrichment using anti-cMyc antibodies (Miltenyi Biotec, 130-092-113) to remove any residual SEM cells. The pure CAR-T cell population was centrifuged, and cell pellets were snap frozen in liquid nitrogen. CD8/CD4 CAR-T cells that were not exposed to SEM (pre-tumor CAR-T cells) were included as well for comparison. Triplicate samples for each condition (pretumor vs post-SEM exposure) for each CAR construct (empty CAR, CD19 CAR, and H24 CAR) were included. RNA extraction, library preparation, and sequencing were performed at BGI. RNA QC was done with Agilent 2100 Bio analyzer and Agilent RNA 6000 Nano kit. Samples were sequenced using the DNB Seq platform from BGI. For genome mapping, clean

reads were mapped to reference genome (hg38) using HISAT. The average mapping ratio to the genome is 95.24% across samples. For gene expression analysis, clean reads were mapped to reference transcripts using Bowtie2 (ver 2.2.5) and expression levels calculated using RSEM (v1.2.8). Gene set enrichment analysis (GSEA) was performed using GSEA software version 4.3.0 (Broad Institute).

CD19 CAR-T relapsed B-ALL PDX establishment. A patient derived xenograft sample from a pediatric patient with pre-B ALL was obtained from the pediatric Hematopoietic Tissue Cell Bank (HTCB) at UCSF. The primary peripheral blood sample was injected via the tail vein into an NSG mouse and passaged once. The HTCB study was approved by the Institutional Review Board at the University of California, San Francisco Benioff Children's Hospital and conducted in accordance with the Declaration of Helsinki.

***Ex vivo* cytotoxicity versus PDX-derived tumor.** Patient derived xenograft (PDX) derived from B-ALL patient who relapsed after CD19 CAR-T cells, established from primary tumor collected under an IRB-approved protocol and in accordance with the Declaration of Helsinki. Flow cytometry was used to determine expression of CD19 and CD72. Tumor cells were cocultured with CAR-T cells at indicated E:T ratios for 24 hours in triplicate. Cytotoxicity was measured by staining for CD72-APC expression. Cytotoxicity at each E:T ratio was calculated with the equation:

% cytotoxicity = (1 - % APC positive tumor cells / average % APC positive tumor cells cultured with untransduced T cells at the corresponding E:T ratio) x 100%

***Ex vivo* cytotoxicity versus primary B-ALL sample.** Primary adult B-ALL specimens were obtained under an IRB-approved protocol and in accordance with the Declaration of Helsinki. Primary B-ALL bone marrow and peripheral blood samples were assessed for CD19, CD22, CD10, CD34 and CD72 expression. Tumor and CAR-T cells were cocultured for 24 hours at the indicated E:T ratios in triplicate. Cells were stained for CD72 and CD3 to determine cytotoxicity. The values were normalized to untransduced cells incubated with tumor. Cytotoxicity at each E:T ratio was calculated with the equation:

% cytotoxicity = (1 - % CD3 negative tumor cells / average % CD3 negative tumor cells cultured with untransduced T cells at given effector to tumor ratio) * 100%

Biolayer Interferometry. Bio-layer interferometry data (BLI) was obtained using an Octet RED384 (ForteBio) instrument. Biotinylated CD72 protein was loaded onto a streptavidin biosensor until 0.2-nm signal was achieved. After blocking with 10 uM biotin, each of the nanobody binders was added to determine binding affinity. PBSTB was used a buffer for all analytes. Data were analyzed using the ForteBio Octet analysis software and kinetic parameters determined with a 1:1 monovalent binding model.

Retrogenix binder specificity studies. The Retrogenix Cell Microarray Technology uses HEK293 transiently transfected to overexpress 6,019 secreted and membrane-tethered proteins. Each construct encoding a plasma membrane protein open reading frame also includes zsGreen as a positive control for transfection. Initial binder specificity screens for nanobody test article "H24-Fc fusion" demonstrated consistent binding to CD72-expressing HEK293 cells. Full microarray library screens ($n = 2$) were performed on fixed HEK293 cells with this test article at a concentration of 10 ug/mL. Live cell confirmation screens ($n = 1$) were performed with comparison to rituximab (1 ug/mL) and no antibody as controls for background and false positive hits. Flow cytometry (gated on zsGreen+, live cells) was used to assess binding to final potential hits of CD72 and CBLIF, with CD20 as control. Standard Retrogenix thresholds of fold-change over background are used to assess true- vs. false-positive binding. Retrogenix determined CD72 as the only specific binding partner of H24-Fc fusion, whereas CBLIF binding was determined to be non-significant and consistent with background noise.

Statistical Analysis. All statistical analyses were performed in GraphPad Prism version 9. Data are presented as mean +/- standard deviation, unless otherwise specified. Statistically significant differences are included in each figure and/or figure legend. All *n* values given are biological replicates, unless otherwise specified. A $p<0.05$ was considered statistically significant and *p*-values were denoted with asterisks as follows (ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). The number of repeats performed, and the statistical tests used, are described in the relevant figure legend.

278 **Data availability.** RNA-seq data is publicly deposited to the Gene Expression Omnibus (GEO)

279 repository with accession number: GSE218791.

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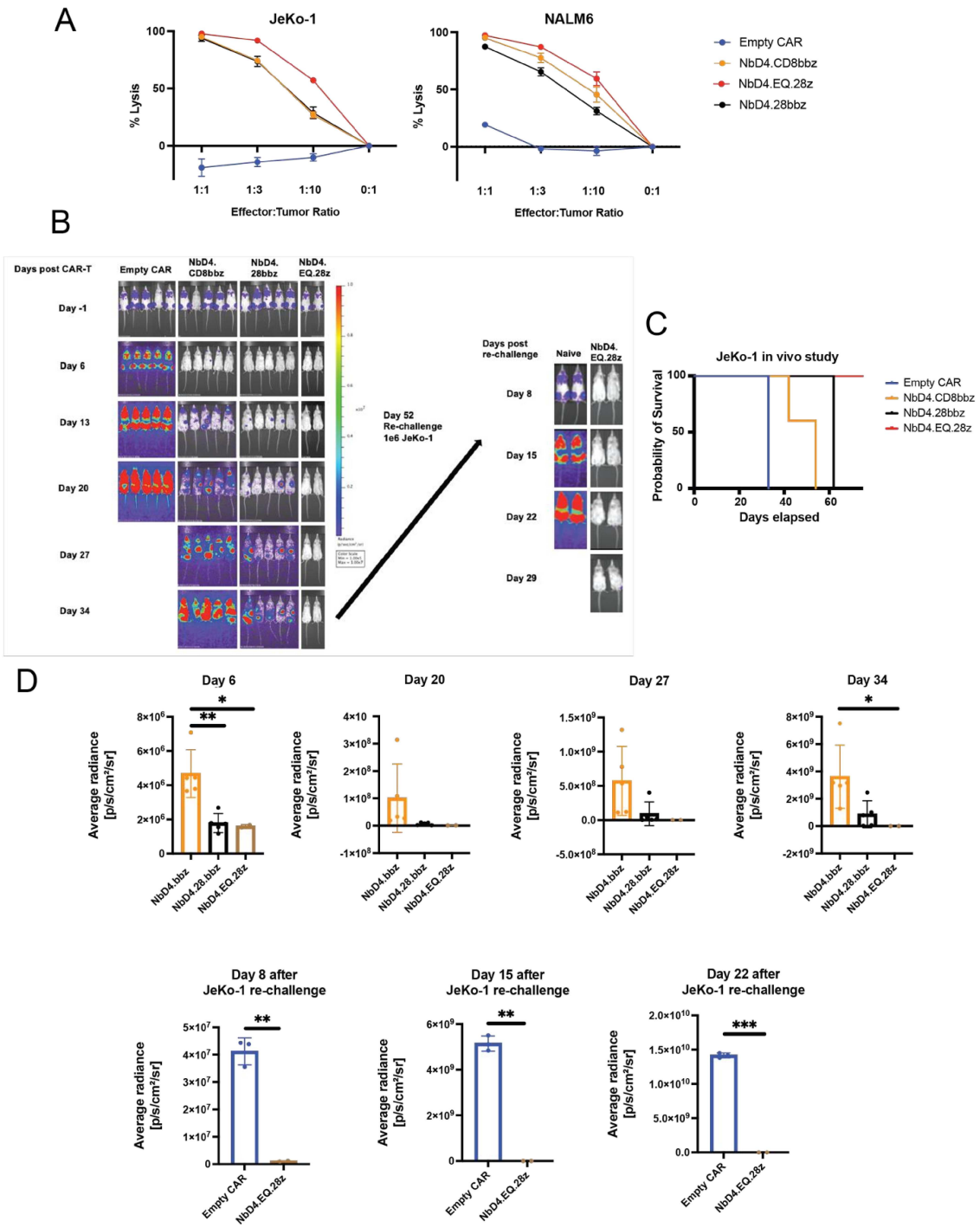
SUPPLEMENTAL FIGURE 1



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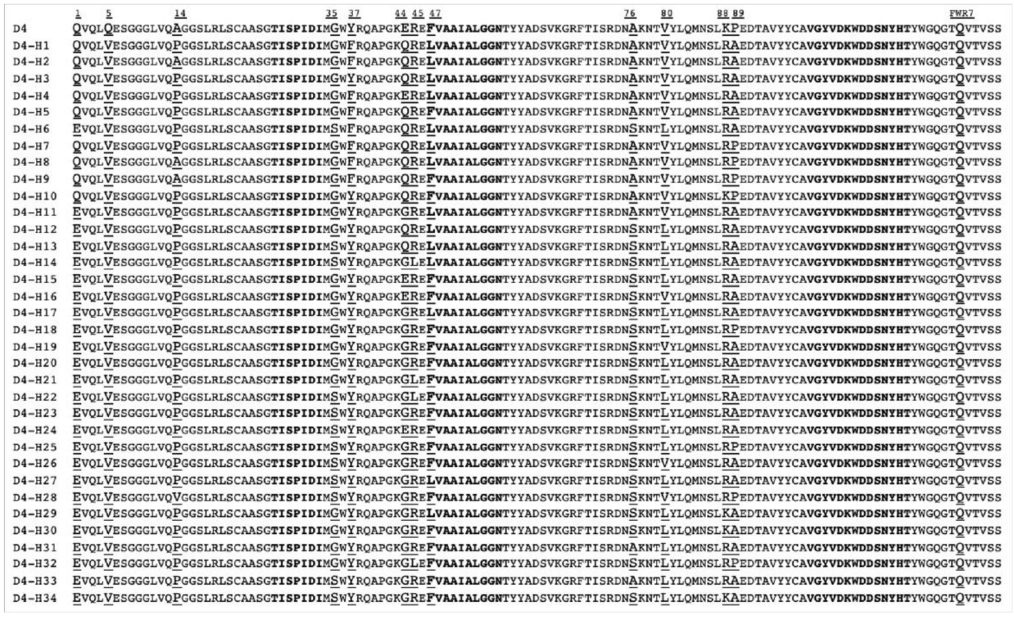
Supplemental Figure 1. NbD4.EQ.28z nanoCARs have potent *in vivo* and *in vitro* anti-lymphoma efficacy compared to other CAR backbones. A. *In vitro* cytotoxicity of NbD4 nanoCARs with various CAR backbones were tested against a B-ALL cell line (NALM6) and a mantle cell lymphoma cell line (JeKo-1) at the indicated effector:tumor ratios, cocultured for 24 hours. Cell viability was measured via bioluminescence. Data was generated from three biological replicates. Error bars were made using the standard error of the mean, and data were normalized to the tumor only (E:T 0:1) condition. **B.** NSG mice were injected with 1e6 Jeko cells on day -4, and on day 0 mice were treated with 4e6 NbD4 CD72 nanoCARs with the various CAR backbones (*n*=5 mice per arm for Empty CAR, NbD4.CD8bbz, and NbD4.28bbz; *n*=2 mice for NbD4.EQ.28z arm). Due to CAR-T manufacturing challenges with the NbD4.EQ.28z construct for this specific study, there were only sufficient CAR-T cells to include two mice in this treatment arm. BLI was performed on day -1 to randomize the mice into different treatment arms to ensure the disease burden was equal across all CAR constructs. JeKo-1 cells express luciferase, and tumor burden was determined using bioluminescent imaging (BLI). At day 52, mice treated with NbD4.EQ.28z nanoCARs, or naïve mice that were not treated (*n*=2 mice per condition), were re-challenged with 1e6 JeKo-1 cells, and disease burden was followed based on weekly BLI. **C.** Kaplan-Meier survival curves are shown for each of the tested nanoCAR constructs. **D.** Tumor burden was assessed weekly via BLI, quantified BLI images on each day after CAR-T injection are shown. Bar graphs represent the mean +/- S.D. Data in **Supplemental Fig. 1C** generated using the long-rank (Mantel-Cox) test. Data in **Supplemental Fig. 1D** generated using an unpaired two-tailed *t*-test. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

SUPPLEMENTAL FIGURE 2

A



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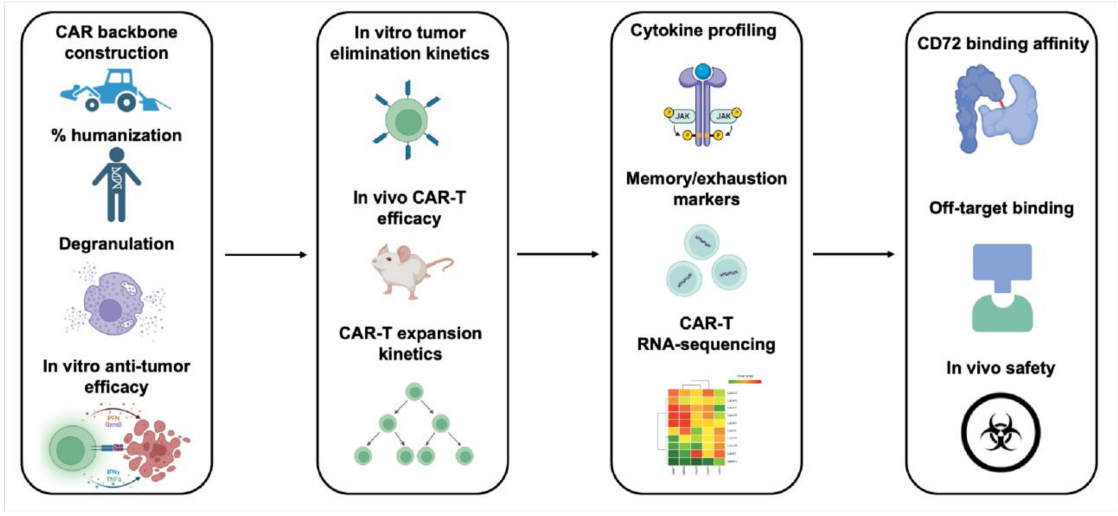

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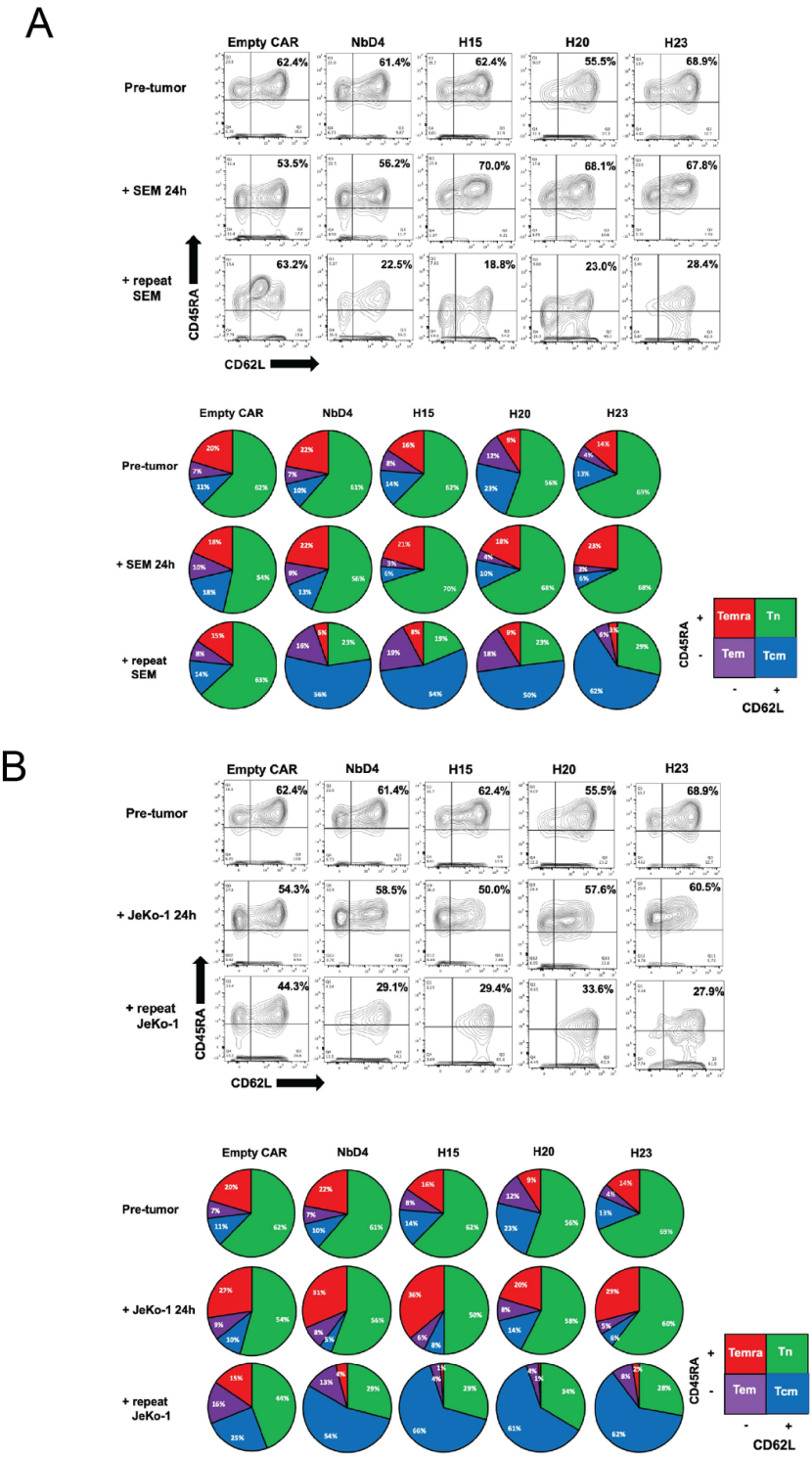
307 **Supplemental Figure 2. Framework humanization of NbD4 and schematic for evaluating**
308 **humanized nanoCARs. A.** Humanization strategy to induce mutations in certain regions of the
309 framework region in the NbD4 construct. Amino acid sequence alignments are shown,
310 comparing parental NbD4 (D4) to 34 humanized NbD4 constructs. Amino acid substitutions
311 were made in the framework regions (specific sites of mutations underlined), while the

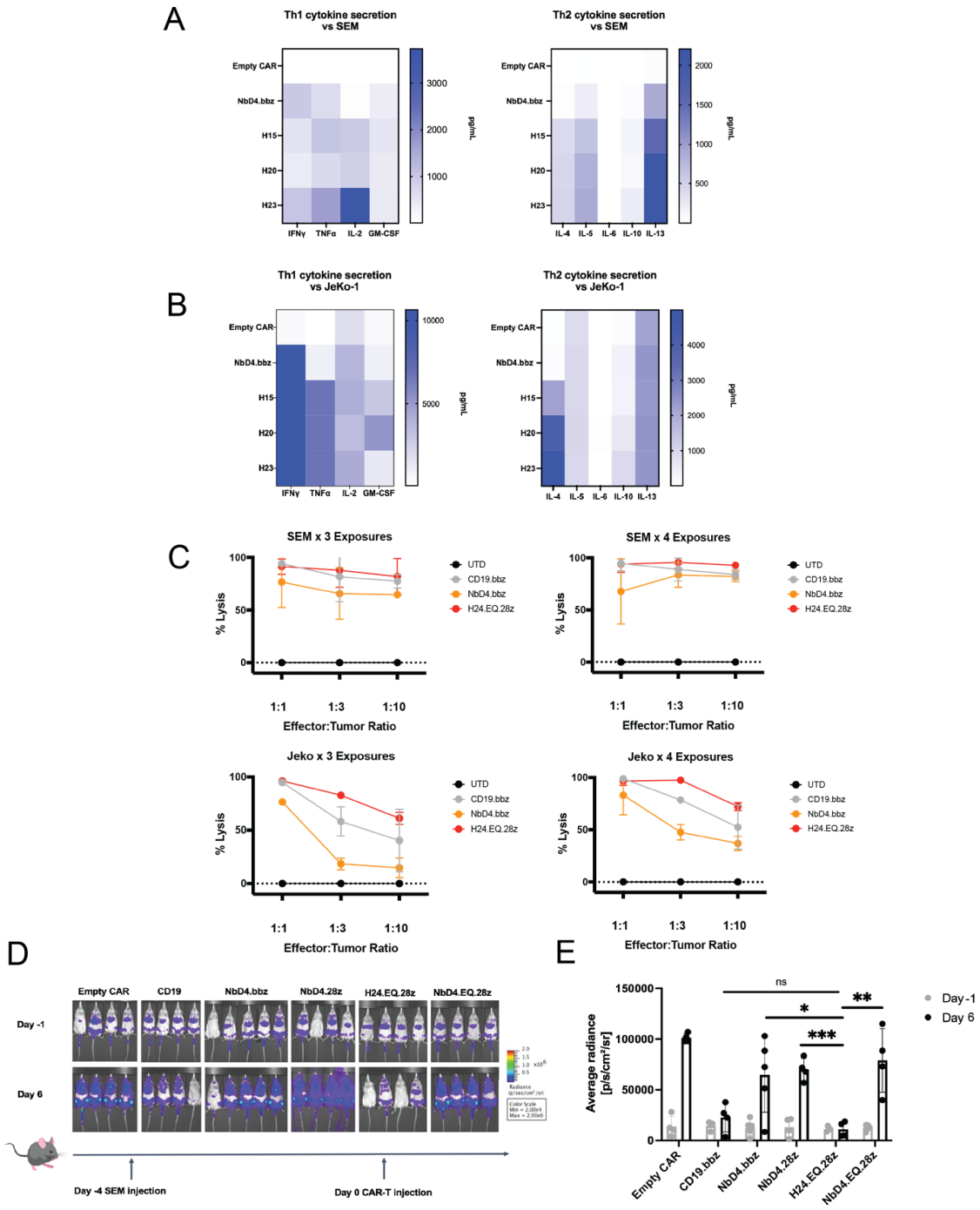
complementarity determining regions (CDRs; in bold) were left unchanged. "Human" comparator V_H sequence is derived from the fully human IgG4 monoclonal antibody trastuzumab. **B.** Rationale and characterization of humanized anti-CD72 nanobody CAR-T cells. Figure made with BioRender.

SUPPLEMENTAL FIGURE 3



Supplemental Figure 3. Memory marker expression on various CD72 nanoCAR constructs decreases with repeated tumor exposure to SEM and JeKo-1. A. Various CD72 nanoCARs and empty CAR-T cells were generated, and flow cytometry was performed to determine cell surface expression of CD45RA and CD62L pre-tumor exposure. Each of the CAR-T cell constructs were then co-cultured with SEM tumors at a 1:1 effector:tumor (E:T) ratio (1e5 SEM cells) for 24 hours, followed by flow cytometry for CD45RA and CD62L. A second exposure with 1e5 SEM tumor cells was added, and 48 hours later flow was performed for CD45RA and CD62L. Circle plots are shown indicating naïve T cells (CD45RA+/CD62L+), central memory T cells (CD45RA-/CD62L+), T effector memory cells re-expressing CD45RA (CD45RA+/CD62L-), and effector memory T cells (CD45RA-/CD62L-). *N* = 1 replicate. **B.** Various CD72 nanoCARs and empty CAR-T cells were generated, and flow cytometry was performed to determine cell surface expression of CD45RA and CD62L pre-tumor exposure. Each of the CAR-T cell constructs were then co-cultured with JeKo-1 tumors at a 1:1 effector:tumor (E:T) ratio (1e5 JeKo-1 cells) for 24 hours, and flow cytometry was done for CD45RA and CD62L. A second exposure with 1e5 JeKo-1 tumor cells was added, and 48 hours later flow was done for CD45RA and CD62L. Circle plots are shown indicating naïve T cells (CD45RA+/CD62L+), central memory T cells (CD45RA-/CD62L+), T effector memory cells re-expressing CD45RA (CD45RA+/CD62L-), and effector memory T cells (CD45RA-/CD62L-). *n* = 1 replicate.

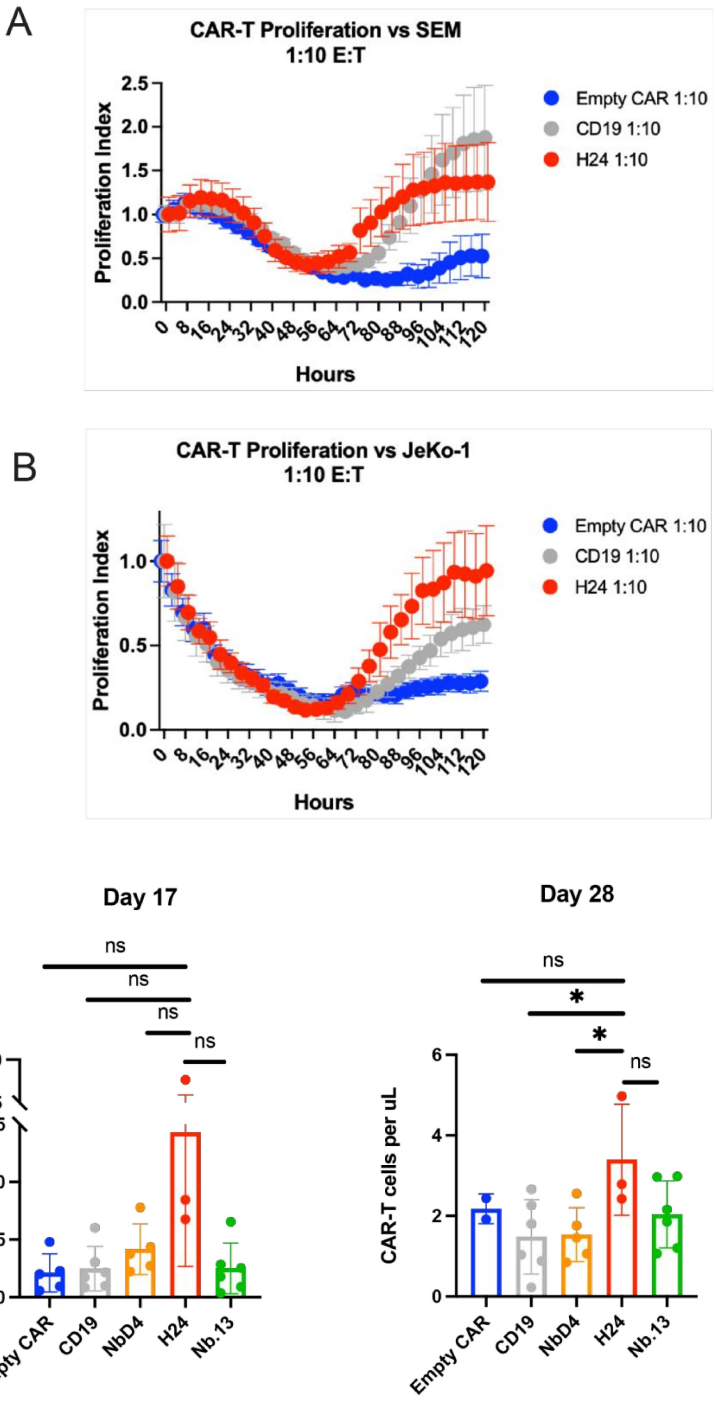
SUPPLEMENTAL FIGURE 4



Supplemental Figure 4. Humanized CD72 nanoCARs have a unique cytokine secretion profile and retain potent anti-tumor efficacy upon multiple tumor exposures. A. CAR-T

cells were co-cultured with SEM tumor cells at a 1:1 E:T ratio for 24 hours. Multiplexed cytokine profiling from culture supernatant after 24-hour SEM exposure. Heatmaps were generated for Th1 or Th2-type cytokine secretion. *n* = 1 replicate. **B.** CAR-T cells were co-cultured with JeKo-1 tumor cells at a 1:1 E:T ratio for 24 hours. Multiplexed cytokine profiling from culture supernatant after 24-hour JeKo-1 exposure. Th1 and Th2 cytokine profiles are shown in the heatmaps. *n* = 1 replicate. **C.** *In vitro* 24-hour cytotoxicity assays comparing H24, NbD4, and CD19 CAR-T cells against SEM or JeKo-1. Every 24 hours, 1e5 SEM or 1e5 JeKo-1 tumor cells were added to each CAR-T cell construct. The third and fourth SEM or JeKo-1 tumor exposures are shown. Data are normalized to untransduced T cells (UTD). *n* = 3 biological replicates. **D.** NSG mice were injected with 1e6 firefly-luciferase labeled SEM B-ALL cells on day -4, and on day 0 mice were treated with a low-dose *in vivo* CAR stress test with 1.5e6 CAR-T cells per mouse (1:2 to 1:4 ratio of CD8/CD4 CAR-T cells). BLI was performed on day -1 to randomize the mice into different treatment arms to ensure the disease burden was equal across all CAR constructs. **E.** Tumor burden was assessed on day -1 and day 6 by BLI, and quantified BLI images are shown. The data from all mice are averaged, and error bars represent standard deviation. Data **Fig. 3E** generated using an unpaired two-tailed t-test. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

SUPPLEMENTAL FIGURE 5



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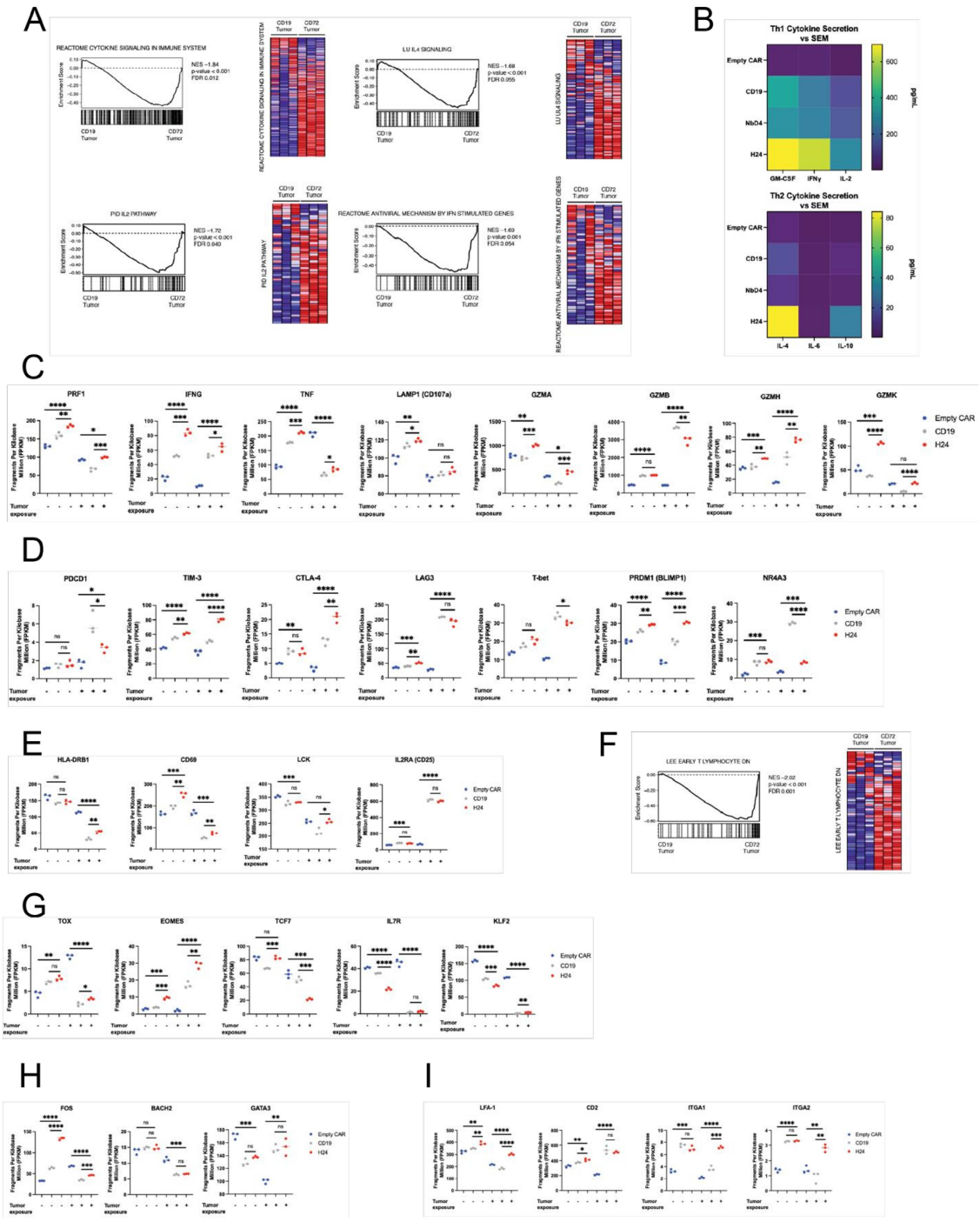
Supplemental Figure 5. H24 nanoCARs have similar in vitro and in vivo proliferation to CD19 CAR-T cells. A. Empty CAR, H24 nanoCARs, and CD19 CAR-T cells were co-cultured with SEM tumor cells at 1:10 E:T ratio for 120 hours; data obtained using Incucyte live-cell imaging (n = 6 technical replicates). **B.** Empty CAR, H24 nanoCARs, and CD19 CAR-T cells were co-cultured with JeKo-1 tumor cells at 1:10 E:T ratio for 120 hours; data obtained using Incucyte live-cell imaging (n = 6 technical replicates). **C.** NSG mice were injected with 1e6 luciferase-labeled SEM B-ALL cells on day -4, and on day 0 mice were treated with 2.5e6 CAR-T cells per mouse. n = 3-6 mice per arm. Peripheral blood was sampled from mice on day 17 and day 28. CAR-T cells were determined based on dual positivity for GFP+ and CD3. Bar graphs represent the mean +/- S.D. Data in **Supplemental Fig. 9C** were generated using an unpaired two-tailed t-test. ns = $p > 0.05$, * = $p < 0.05$.

SUPPLEMENTAL FIGURE 6



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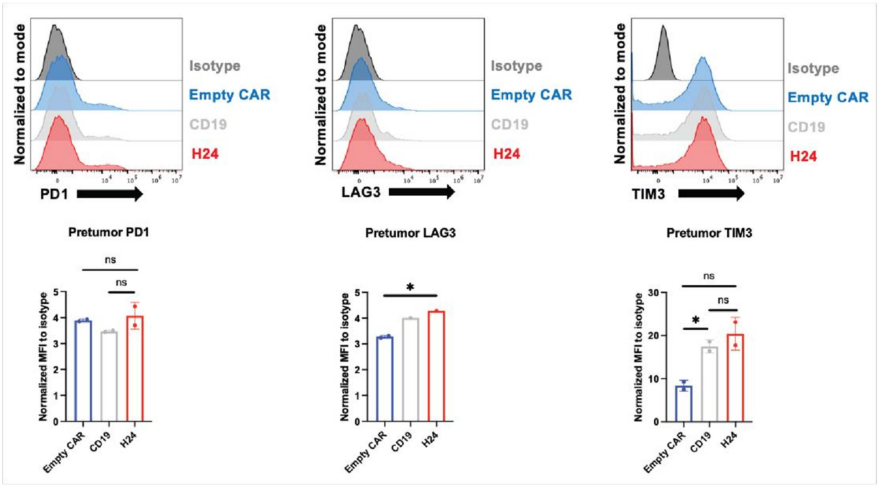
Supplemental Figure 6. RNA-seq reveals that H24 nanoCARs activate unique transcriptional pathways, compared to CD19 CAR-T cells, before and after exposure to SEM tumors. A. Gene set enrichment analysis (GSEA) plots and heatmaps showing different transcriptional pathways involved in cytokine signaling that are upregulated in H24 nanoCARs compared to CD19 CAR-T cells, after exposure to SEM for 24 hours. FDR = false discovery rate. **B.** Empty CAR, CD19, NbD4, and H24 CAR-T cells were co-cultured with SEM tumors for 24 hours. Multiplexed cytokine profiling from culture supernatant after 24-hour SEM exposure. Heatmaps were generated for Th1 or Th2-type cytokine secretion. Experiment performed in biological triplicate. **C.** Expression of selected cytokines and genes involved in cytotoxicity, before and after exposure to SEM. **D.** Expression of selected inhibitory receptors, before and after exposure to SEM. **E.** Expression of selected genes involved in T cell activation, before and after exposure to SEM. **F.** GSEA plot and heatmap showing a transcriptional pathway involved in early T cell differentiation, after exposure to SEM for 24 hours. **G.** Expression of selected genes involved in stem-like CAR-T cell characteristics, before and after exposure to SEM. **H.** Expression of genes shown to be upregulated in patients with long CAR-T cell persistence greater than six months, before and after exposure to SEM. **I.** Expression of genes important for cell-cell adhesion that are involved in CAR-T elimination of tumor cells, before and after exposure to SEM. Data in **Supplemental Fig. 5C-E; Supplemental Fig. 5G-I** were generated using an unpaired two-tailed t-test. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

SUPPLEMENTAL FIGURE 7

A



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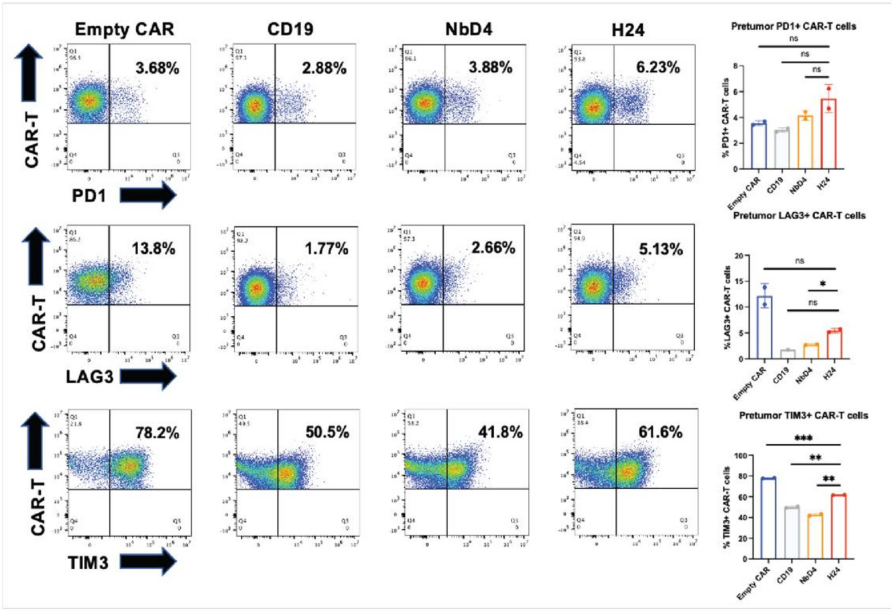
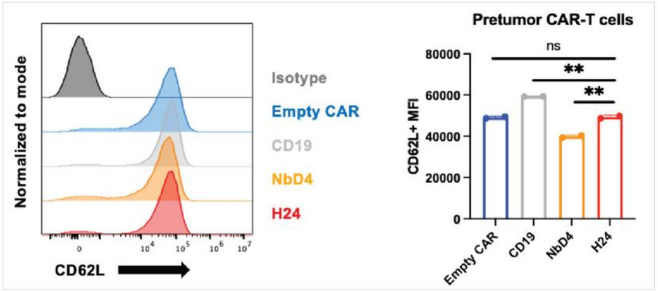


C



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Supplemental Figure 7. Pretumor exhaustion marker profiling reveals that H24 nanoCARs have similar expression compared to other CAR-T constructs. A. H24, CD19, and empty CAR-T cells were tested for baseline levels of the exhaustion markers PD1, LAG3, and TIM3 by flow cytometry. The mean fluorescence intensity (MFI) was normalized to each isotype control, and bar graphs are shown for each exhaustion marker. Experiment performed in duplicate. Bar graphs represent the mean +/- S.D. **B.** H24, NbD4, CD19, and empty CAR-T cells were tested for baseline levels of the exhaustion markers PD1, LAG3, and TIM3 by flow cytometry, using a different T cell donor compared to **Supplemental Fig. 6A**. Expression of CAR-T cells that are positive for each exhaustion marker is listed in the upper right quadrant. Experiment performed in duplicate. Bar graphs represent the mean +/- S.D. **C.** H24, NbD4, CD19, and empty CAR-T cells were tested for baseline levels of the memory marker CD62L by flow cytometry. Bar graphs represent the mean +/- S.D. Data in **Supplemental Fig. 6A-C** were generated using an unpaired two-tailed t-test. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

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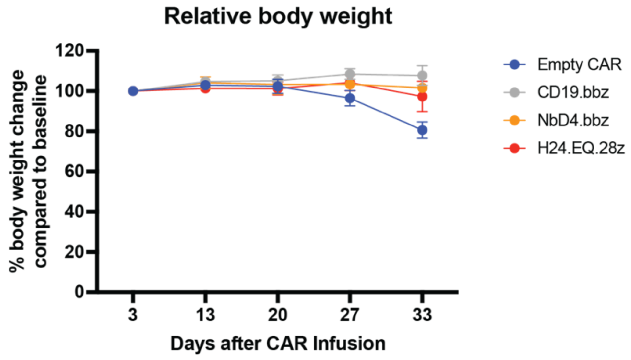
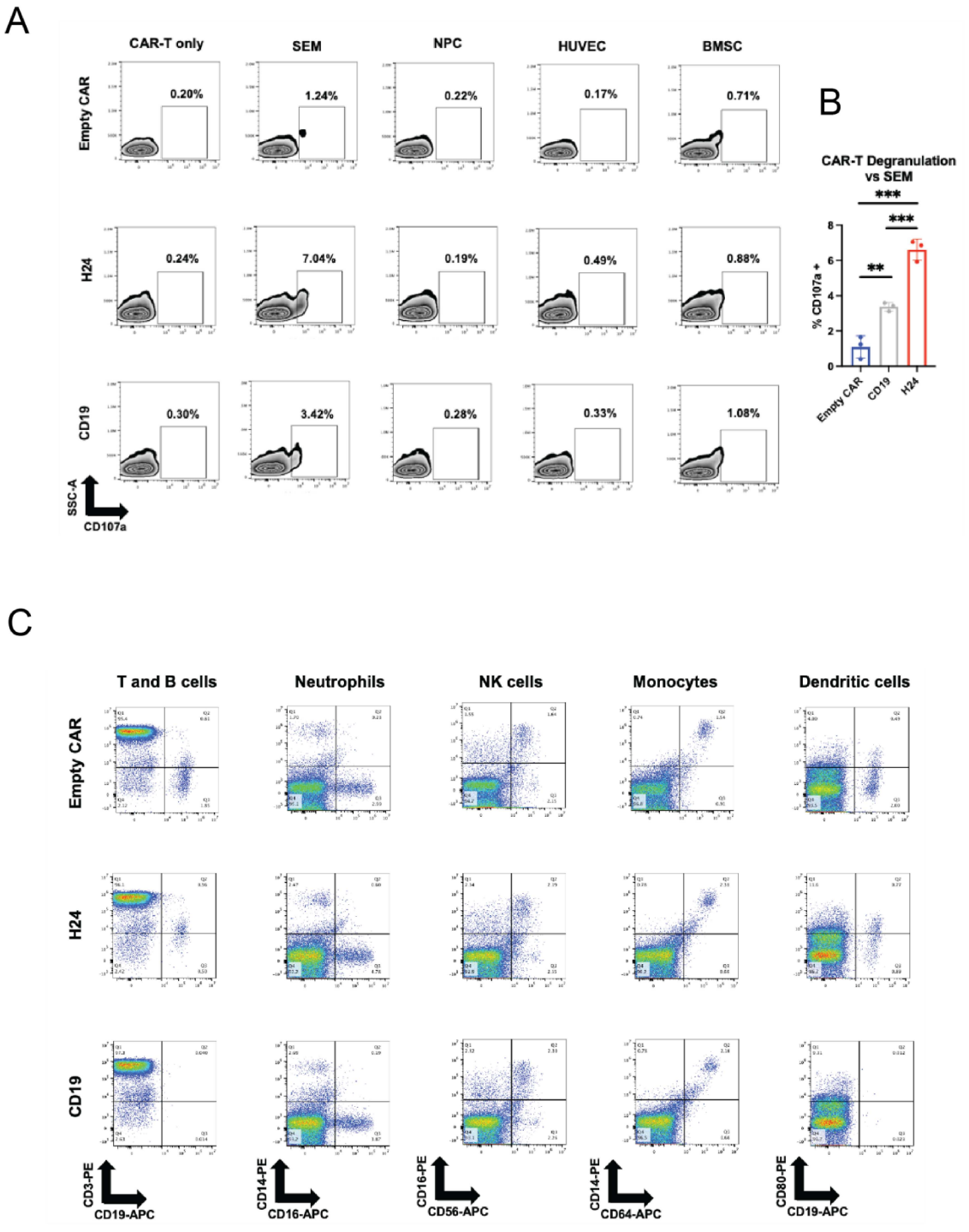
SUPPLEMENTAL FIGURE 8



422

423 **Supplemental Figure 8. Relative mouse body weight over the course of the *in vivo***
424 **lymphoma study in Figure 5**. NSG mice were injected with 5e5 firefly-luciferase labeled JeKo-
425 1 mantle cell lymphoma cells on day -4, and on day 0 mice were treated with 3e6 CAR-T cells
426 per mouse (n = 5/arm, as shown). During the study, mice weights were obtained once per week.
427 Mouse weights were normalized to the initial weight that was obtained on day 3.

SUPPLEMENTAL FIGURE 9



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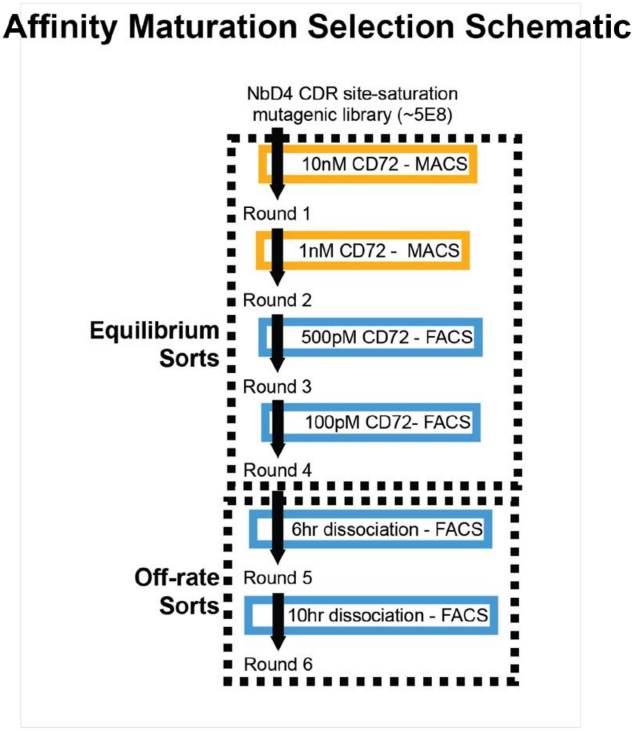
Supplemental Figure 9. H24 nanoCARs have a reassuring toxicity profile against normal human tissues. A. *In vitro* flow cytometry-based assay to determine CAR-T degranulation against different target cells using CD107a. H24, CD19, and empty CAR-T cells were generated and co-cultured at a 2:1 E:T ratio against the indicated cell types for six hours. SEM = *KMT2A*r B-ALL cell line (n = 3 technical replicates); NPC = neuronal progenitor cells (n = 3 technical replicates); HUVECS = human umbilical vein endothelial cells (n = 1); BMSC = bone marrow stromal cells (n = 1). Percentage of CAR degranulation is displayed. Bar graph shows CD107a expression against the CD19+/CD72+ SEM cell line. Bar graphs represent the mean +/- S.D. **B.** Flow cytometric analysis of normal donor peripheral blood mononuclear cells (PBMCs) after 24-hour co-culture with either H24, CD19, or empty CAR-T cells. Flow plots display lack of cell ablation for T-cells, neutrophils, NK cells, monocytes, and dendritic cells. B-cells were fully eradicated by CD19 CAR-T cells while partly eradicated by H24 nanoCARs. Experiment performed in duplicate. Data in **Supplemental Fig. 7B** were generated using an unpaired two-tailed t-test. ** = $p < 0.01$, *** = $p < 0.001$

SUPPLEMENTAL FIGURE 10

A



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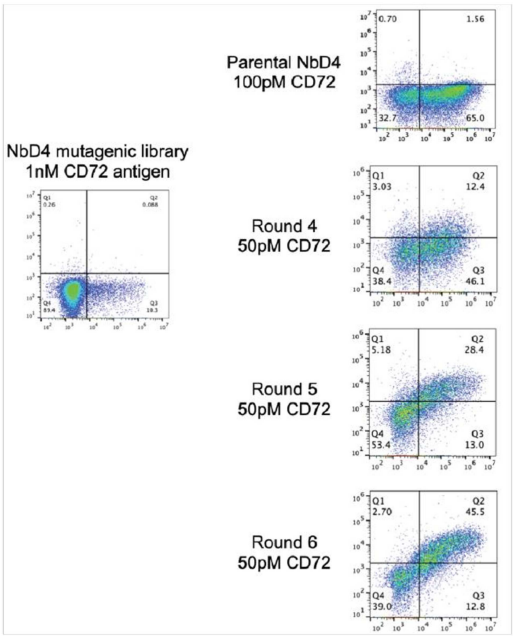

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Supplemental Figure 10. Affinity maturation of CD72 nanobody binders. A. Affinity maturation selection schematic. **B.** Six total rounds of MACS and FACS selections were performed with a CDR site saturated NbD4 yeast display library to isolate variants with increased binding affinity for CD72. Four equilibrium sorts of increasing stringency were performed, followed by two high-stringency kinetic off-rate sorts of 6-hrs and 10-hrs with FACS gating on the top 1% of binding clones. *y*-axis of flow cytometry plots represents FITC-conjugated recombinant CD72 bound to yeast; *x*-axis represents HA staining of yeast successfully expressing a surface nanobody.

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Antibody, clone	**Manufacturer**	**Catalog number**
Human CD72-APC (Clone 3F3)	Biolegend	316210
Human CD19-APC (Clone HIB19)	BD Biosciences	555415
Human CD19-PE (Clone HIB19)	Biolegend	302208
Human CD3-APC (Clone SK7)	Biolegend	344812
Human CD3-PE (Clone UCHT1)	Biolegend	300456
Human CD22-FITC (Clone HIB22)	Biolegend	302504
Human CD34 Pacific Blue (Clone 581)	Biolegend	343512
Human CD10 (Clone HI10a)	Biolegend	312230
Human CD107a-APC (Clone H4A3)	BD Biosciences	560664
Human CD45RA-PE (Clone HI100)	Invitrogen	12-0458-42
Human CD62L-APC (Clone DREG-56)	BD Biosciences	559772
Human PD1-APC (Clone EH12.2H7)	Biolegend	329908
Human LAG3-APC (Clone 3DS223H)	Invitrogen	17-2239-41
Human TIM3-APC (Clone F38-2E2)	Invitrogen	17-3109-42
Alexa Fluor 647 Streptavidin	Biolegend	405237
Human CD16-APC (Clone 3G8)	Biolegend	302011

Human CD14-PE (Clone 63D3)	Biolegend	367103
Human CD64-APC (Clone 10.1)	Biolegend	305013
Human CD80-PE (Clone 2D10)	Biolegend	305208

Supplemental Table 1. List of all antibodies used for flow cytometry.

456 B-ALL pediatric patient derived xenograft:

PDX name	**Mutation**	**Treatment status**	**Cytogenetics**	**Age**	**Sex**
HM5566	*TP53* p.R280T, *MYC* amplification, *CDKN2A* and *CDKN2B* deep deletion	Relapse after CD19 CAR-T cells	ETV6-RUNX1 rearrangement	Patient is less than 10 years old	female

457

458

459 B-ALL adult primary patient samples:

Sample name	**Mutation**	**Cytogenetics**	**Treatment Status**	**Sex**
HMTB00911	*CDKN2A* and *CDKN2B* loss	45-46,X,-X,add(5)(q11.2),del(5)(q22q33), del(9)(p13),add(11)(q23), ?del(16)(q22)[11]/46,XX[17].nuc ish(ABL1,BCR)x2[173]	Relapse after CD19 CAR-T cells	female
HMTB00716	*KMT2A*r, *MYC* rearrangement	46XX	Relapse after CD19 CAR-T cells	female

460

461 **Supplemental Table 2.** Patient characteristics of the patient derived xenograft (PDX) and the

462 two primary patient samples that were used in **Fig. 3D-I**.

463

464

SUPPLEMENTAL TABLE 3

Organ System	Empty CAR (H&E)	Empty CAR (IHC for CD3 & CD72)	CD19 (H&E)	CD19 (IHC for CD3 & CD72)	H24 (H&E)	H24 (IHC for CD3 & CD72)
Liver	NSF	NSF	Cellular infiltrate, mononuclear (large cell), rare	Cellular infiltrates negative for CD72 and CD3	Cellular infiltrate, mononuclear (large cell), multifocal, mild	Nodular cellular infiltrate negative for CD3 and CD72
Spleen	Nodular cellular infiltrate (presumed lymphoid, large cell), multifocal, mild to moderate	CD72: Scattered CD72+ cells; CD3: Absence of CD3+ cells. Nodular aggregates negative for CD72 and CD3.	Nodular cellular infiltrate (presumed lymphoid, large cell), multifocal mild	CD72: Scattered CD72+ cells; CD3: Absence of CD3+ cells	Nodular cellular infiltrate (presumed lymphoid, large cell), multifocal, moderate	CD72: Scattered CD72+ cells; CD3: Absence of CD3+ cells
Kidney	NSF	NSF	NSF	NSF	NSF	NSF
Adrenal gland	NSF	NSF	NSF	NSF	NSF	NSF
Pancreas	NSF	NSF	NSF	NSF	NSF	NSF
Lung	Minimal, alveolar histiocytosis	NSF	Minimal, alveolar histiocytosis	NSF	Minimal, alveolar histiocytosis	NSF
Heart	NSF	NSF	NSF	NSF	Endocardiosis, mild	NSF
Stomach	NSF	n/a	NSF	n/a	NSF	n/a
Small Intestine	NSF	n/a	NSF	n/a	NSF	n/a
Large Intestine	Mural hematoma; protozoa	n/a	Protozoa	n/a	Protozoa	n/a
Brain	NSF	NSF	NSF	NSF	NSF	NSF
Reproductive tract	NSF	n/a	NSF	n/a	NSF	n/a
Lower urinary tract	NSF	n/a	NSF	n/a	NSF	n/a
Skin	NSF	n/a	NSF	n/a	NSF	n/a

465

466 **Supplemental Table 3. Summary autopsy and immunohistochemistry (IHC) evaluation for**
467 **mice who received H24 nanoCARs, CD19 CAR-T cells, or empty CAR-T cells.** NSG mice
468 were injected with 1e6 luciferase-labeled SEM B-ALL cells on day -4, and on day 0 mice were
469 treated with 3e6 CAR-T cells per mouse. *n* = 2 mice per arm. On day 14, mice were sacrificed
470 and H&E staining was done on all major organs was performed as part of a full murine autopsy.
471 Immunohistochemistry against human CD3 was used to determine CAR-T infiltration in murine
472 tissues, and CD72 to determine the presence of SEM tumor cells in different murine tissues. The
473 table represents H&E and IHC results from the study, stratified by each organ system. Summary
474 histopathologic findings from the two mice from each CAR-T treatment arm are reported in the
475 table. A murine pathologist was blinded to the study design and determined that the following
476 histopathologic features were incidental findings: the presence of protozoa in the intestinal tract
477 (cecum), minimal alveolar histiocytosis, intestinal mural hematoma, focal renal tubule dilation,
478 and a single instance of mild endocardiosis. *n* = 2 mice for each CAR treatment arm. NSF = no
479 significant findings. N/A = not available.